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REPARACIÓN DE FIBRAS DE SUSTANCIA BLANCA TRAS INFARTO CEREBRAL SUBCORTICAL. EFECTO DE LA ADMINISTRACIÓN DEL FACTOR NEUROTRÓFICO DERIVADO DE CEREBRO Y DE CÉLULAS TRONCALES

JAIME RAMOS CEJUDO

Madrid 2015

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Tesis Doctoral presentada por el licenciado

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para optar al grado de **DOCTOR**

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CERTIFICAN:

Que el presente trabajo titulado "**REPARACIÓN DE FIBRAS DE SUSTANCIA BLANCA TRAS INFARTO CEREBRAL SUBCORTICAL. EFECTO DE LA ADMINISTRACIÓN DEL FACTOR NEUROTRÓFICO DERIVADO DE CEREBRO Y DE CÉLULAS TRONCALES**" ha sido realizado por Don Jaime Ramos Cejudo bajo su dirección y se encuentra en condiciones de ser leído y defendido como Tesis para alcanzar el grado de Doctor ante el Tribunal correspondiente en la Universidad Autónoma de Madrid.

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ABREVIATURAS Y ACRÓNIMOS

Relación de abreviaturas que aparecen en el texto. En muchos casos se ha conservado la correspondiente abreviatura en inglés debido a su frecuente utilización en el lenguaje científico.

µl: microlitro
µm: micrómetro
ºC: grados centígrados
A2B5: marcador de células precursoras de oligodendrocitos
ACM: arteria cerebral media
ADC: coeficiente de difusión aparente
ADN: ácido desoxirribonucleico
AIT: ataque isquémico transitorio
ANOVA: análisis de la varianza
APC (CC1): marcador de células precursoras de oligodendrocitos
ATP: adenosín trifosfato
B-Actina: proteína beta-actina
BCA: método del ácido bicinconínico
BDNF: factor neurotrófico derivado del cerebro (del inglés *brain derived neurotrophic factor*)
bFGF: factor de crecimiento de fibroblasto básico (del inglés *basic fibroblastic growth factor*)
BHE: barrera hematoencefálica
BSA: albúmina de suero bovina
cm^2: centímetros cuadrados
CMH: complejo mayor de histocompatibilidad
CNPase: 2', 3'-cyclic-nucleotide 3'-phosphodiesterase
$C0_2$: dióxido de carbono
CPO: célula precursora de oligodendrocitos
CTE: células troncales embrionarias
CTH: células troncales hematopoyéticas
CTM: células troncales mesenquimales
CTM-MO: células troncales mesenquimales derivadas de médula ósea
CTN: células troncales neurales
CTM-TA: células troncales mesenquimales derivadas de tejido adiposo
d: días
DAB: diaminobenzidina

DAPI: *4',6-diamidino-2-phenylindole*

DCX: marcador doblecortina

DiI: 1,1 '-dioctadecil-3, 3,3', 3'-tetramethylindocarbocyanine perclorato

DMSO: dimetilsulfóxido

ECL: quimioluminiscencia enzimática (del inglés *enzymatic chemiluminiscence*)

ECV: enfermedad cerebrovascular

EDTA: ácido etilendiaminotetraacético

EEF: escala de evaluación funcional

ELISA: *enzyme linked immunoabsorbant assay*

ET-1: endotelina-1

FC: frecuencia cardiaca

FITC: isotiocianato de fluoresceína

FSC: flujo sanguíneo cerebral

g: gramos

G-CSF: factor estimulante de colonias de granulocitos (del inglés *granulocyte colony-stimulating factor*)

GFAP: proteína ácida fibrilar glial (del inglés *glial fibrillary acidic protein*)

h: horas

HGF: factor de crecimiento de hepatocitos (del inglés *hepatocyte growth factor*)

HRP: peroxidasa de rábano (del inglés *horseradish peroxidase*)

IC: infarto cerebral

i.c.: intracarotídea

IGF: factor de crecimiento de insulina (del inglés *insulin-like growth factor*)

i.p.: intraperitoneal

ITD: imagen de tensor de difusión

i.v.: intravenosa

KI67: antígeno de proliferación celular

l: litro

MBP: proteína básica de mielina (del inglés *myelin basic protein*)

min: minutos

ml: mililitro

NeuN: marcador neuronal

NF: neurofilamento

NGF: factor de crecimiento nervioso (del inglés *nerve growth factor*)

Nogo-A: factor inhibidor de crecimiento axonal (del inglés *neurite outgrowth inhibitor*)

O4: marcador de oligodendrocitos

OACM: oclusión de la arteria cerebral media

Olig-2: factor de transcripción 2 de oligodendrocitos

OMS: Organización Mundial de la Salud

PA: presión arterial

PBS: tampón fosfato salino (del inglés *phosphate buffered saline*)

PDGFR: factor de crecimiento derivado de plaquetas

PE: ficoeritrina

PF: parámetros fisiológicos

pg/mL: picogramo/mililitro

RM: resonancia magnética

r.p.m.: revoluciones por minuto

rtPA: activador de plasminógeno tisular recombinante

SDF-1: factor-1 derivado de células estromales (del inglés *stromal cell-derived factor-1*)

SNC: sistema nervioso central

STEPS: *Stem Cell Therapies as an Emerging Paradigm in Stroke*

SYP: sinaptofisina

TBS: tampón Tris salino (del inglés *tris buffered saline*)

TdT: *terminal deoxynucleotidyl transferase*

TNF-alpha: factor de necrosis tumoral alfa (del inglés *tumor necrosis factor*)

TUNEL: *TdT-mediated dUTP nick end labelling*

u.a: unidades arbitrarias

VEGF: factor de crecimiento del endotelio vascular (del inglés *vascular endothelial growth factor*)

ZSV: zona subventricular

RESUMEN

RESUMEN

Introducción

Habiendo sido demostrada la eficacia del tratamiento reperfusor en el infarto cerebral, del que únicamente pueden beneficiarse un número limitado de pacientes, la investigación se orienta a la búsqueda de nuevas estrategias terapéuticas enfocadas a la potenciación de la reparación cerebral. La recuperación tras el ictus es un proceso complejo y dinámico, que incluye respuestas frente al daño en las zonas de lesión, así como procesos de reparación activos que deben ser debidamente activados para la correcta remodelación tisular. En los últimos años ha quedado demostrado experimentalmente en modelos animales de infarto cerebral cómo estos mecanismos pueden ser potenciados mediante la administración de factores tróficos y terapia celular. En la mayoría de estos estudios la lesión se circunscribe a la corteza, por lo que todavía no hay evidencias suficientes de si dichas terapias son capaces de potenciar la reparación de las fibras de sustancia blanca subcorticales tras el ictus. A pesar de que la sustancia blanca ocupa cerca del 50% del volumen cerebral total, y que la afectación subcortical en los pacientes de isquemia es frecuente tanto en oclusiones de gran vaso como en las de pequeño vaso, la mayoría de estudios no se han ocupado de ella, por lo que es fundamental incorporar nuevos modelos animales de infarto cerebral subcortical, que sirvan como herramientas para estudiar los mecanismos subyacentes al daño y reparación de dichas fibras nerviosas (axón y mielina).

Entre los factores tróficos que se han estudiado en modelos animales de infarto cerebral cortical, destacan el factor de crecimiento de fibroblastos (bFGF), el factor de crecimiento endotelial-vascular (VEGF) y el factor neurotrófico derivado del cerebro (BDNF), siendo éste último uno de los que más interés ha suscitado en los últimos años, gracias a su papel en la supervivencia neuronal, la formación y diferenciación de nuevas neuronas, el aprendizaje y la memoria. Puesto que estudios recientes han mostrado que el BDNF participa en el mantenimiento y supervivencia de las fibras nerviosas, así como promueve la formación de nuevas estirpes neurales, sería de interés estudiar si su

administración terapéutica pudiera tener la capacidad de promover la reparación de las fibras nerviosas de sustancia blanca tras infarto cerebral de tipo subcortical.

Respecto a la terapia celular, múltiples estirpes celulares se han ensayado en modelos animales, entre las que destacan las células troncales neurales, las de cordón umbilical, así como las células troncales mesenquimales derivadas de médula ósea o de tejido adiposo (CTM-TA). Estas últimas son de especial interés, dada su abundancia y fácil obtención, además de no presentar conflictos de tipo ético. Estudios con CTM-TA en modelos animales de infarto cerebral cortical, han demostrado eficacia sobre la recuperación funcional, la disminución del tamaño de lesión, así como la muerte celular. Sin embargo, los mecanismos de acción subyacentes son aún desconocidos. Trabajos recientes han demostrado la capacidad de las CTM-TA para promover una modulación de los niveles de marcadores de reparación cerebral tras infarto cortical, por lo que sería de interés estudiar si las CTM-TA pudieran tener un efecto positivo sobre la reparación de fibras tras infarto cerebral subcortical.

Hipótesis

Los mecanismos de reparación en el infarto cerebral cortical pueden ser potenciados mediante la administración de factores tróficos, así como mediante la terapia celular. Sin embargo, todavía no ha sido suficientemente demostrado si dichas terapias son capaces de potenciar la reparación de las fibras de sustancia blanca tras infarto cerebral subcortical. En este sentido, es posible que la administración del factor neurotrófico derivado del cerebro (BDNF) así como, la administración de células troncales mesenquimales derivadas de tejido adiposo (CTM-TA) tras infarto subcortical pueda ser capaz de potenciar la reparación de las fibras de sustancia blanca, mejorando la conectividad cerebral y la recuperación funcional.

Objetivos

En un modelo experimental en rata de infarto cerebral subcortical con afectación de sustancia blanca pretendemos:

1. Analizar el efecto de la administración intravenosa del Factor Neurotrófico derivado del cerebro (BDNF) sobre la recuperación funcional.
2. Estudiar el efecto de la administración intravenosa de BDNF sobre la reparación de las lesiones de sustancia blanca, la proliferación celular, crecimiento de fibras y recuperación de la conectividad cerebral.
3. Analizar el efecto de la administración intravenosa de células troncales mesenquimales derivadas de tejido adiposo (CTM-TA) sobre la recuperación funcional.
4. Estudiar el efecto de la administración intravenosa de CTM-TA sobre la proliferación y diferenciación de células precursoras de oligodendrocitos (CPO), la reparación de las lesiones de sustancia blanca, el crecimiento axonal, así como la recuperación de la conectividad cerebral.

Material y métodos:

Se utilizaron un total de 146 ratas macho Sprague-Dawley (200-250g). Para el estudio del tratamiento con BDNF, los animales fueron distribuídos en 3 grupos: 1) sham (n=24); 2) Control (n=24); 3) BDNF (n=24). 2 animales fueron excluídos del estudio, puesto que uno de los animales falleció durante el procedimiento quirúrgico y otro durante la resonancia magnética. Para el estudio del tratamiento con CTM-TA, los animales fueron distribuídos en: 1) sham (n=24); 2) Control (n=24); 3) CTM-TA (n=24). En ambos estudios, los animales del grupo sham fueron sometidos a todo el procedimiento quirúrgico a excepción de la inyección de Endotelina-1 (ET-1) a nivel subcortical y se administró suero salino por vía i.v como tratamiento. El resto de animales fueron sometidos a la inyección de ET-1 en las coordenadas estereotácticas +0,4mmAP, +3,5mmL, + 6mmDV respecto al

área de Bregma, y se les administró suero salino (grupo control), BDNF (100µg), o CTM-TA ($2x10^6$ células) por vía i.v. En ambos estudios los animales se sacrificaron a los 7 y 28 días (n=10 en cada tiempo). Para los estudios de biodistribución los animales fueron sacrificados a las 4h (BDNF; n=4) o a las 24h (CTM-TA; n=4), junto con sus respectivos controles (n=4 para cada estudio).

Tras la obtención de CTM-TA de ratas adultas Sprague-Dawley, se llevó a cabo el aislamiento y caracterización de las células por citometría de flujo. Se analizó la evaluación funcional mediante el test de la barra longitudinal, el del cilindro rotarorio y el test de Rogers, a tiempo basal (antes de las cirugía), a las 24 y 72h tras el tratamiento, así como a los 7 y 28 días. Se estudió la biodistribución de los tratamientos (CTM-TA y BDNF) en el cerebro, así como en órganos periféricos. Además, se analizó el tamaño de lesión mediante Resonancia Magnética (RM) de 7 Teslas. Se obtuvieron mapas de difusión y se analizó la conectividad cerebral en los animales mediante tractografías a las 24h, 7 y 28 días tras el tratamiento. A los 7 y 28 días se estudió: la integridad de las fibras de mielina mediante la técnica Cryomyelin; la muerte celular por la técnica TUNEL; la proliferación celular mediante el marcador KI67; comarcajes de KI67 con marcadores neuronales (NeuN), gliales (GFAP), de oligodendrocitos (Olig-2) y de células precursoras de oligodendrocitos (CPO) como A2B5, CNPase, APC (CC1) y PDGFR. Además, se analizaron los niveles de marcadores asociados a reparación de sustancia blanca como la proteína neurofilamento (NF), la proteína básica de mielina (MBP), el marcador 4 de oligodendrocitos (O4) y la proteína inhibidora de crecimiento axonal Nogo-A mediante inmunofluorescencia y Western blot. Finalmente, con el fin de identificar el conjunto de factores secretados al medio extracelular por las CTM-TA, una vez expandidas in vitro, las células se cultivaron en medio libre de proteínas durante 24h al tercer pase (P3). Transcurrido este tiempo se aislaron y filtraron los sobrenadantes para los estudios de proteómica por Orbitrap. Las proteínas identificadas fueron clasificadas según sus funciones mediante estudios de ontología genética (Gene Ontology del inglés).

Resultados

Efecto del tratamiento con BDNF tras infarto subcortical El tratamiento con BDNF mostró una buena recuperación funcional en comparación con el grupo control en el test de la barra longitudinal, el test del cilindro rotatorio y el test de Rogers a los 28 días (P<0.05). El grupo sham no mostró déficit en ninguna de las evaluaciones. Al analizar la biodistribución del BDNF, se detectó aumento significativo de los niveles de BDNF en el cerebro de los animales a las 4h de la administración intravenosa detectado mediante ELISA, Western blot e immunofluorescencia (P<0.05). El estudio de RM no reveló diferencias significativas en el tamaño de lesión ni a las 7 ni a los 28 días entre el grupo control y el tratado con BDNF. Sin embargo, al analizar las imágenes de tractografía se observó una mejoría en la conectividad cerebral en el grupo de animales tratados con el factor trófico en comparación con el grupo control a los 28 días, lo cual estaba asociada a un mayor marcaje de mielina en el estudio histológico por la técnica de Cryomyelin (P<0.05). Se observó un incremento en la proliferación celular en el grupo de animales tratados con el factor trófico a los 7 días en comparación con el grupo control (P<0.05). Las células positivas para el marcador de proliferación KI67 colocalizaron con marcadores de CPO tales como A2B5, CNPase, APC (CC1) y PDGFR. Al analizar los niveles globales de marcadores asociados a CPO a los 7 días, se observó un aumento en los niveles cerebrales de los marcadores A2B5, CNPase y O4 en los animales tratados con BDNF en comparación con el grupo control (P<0.05). Finalmente, al analizar los marcadores asociados a reparación de sustancia blanca a los 28 días tras el tratamiento, se observó un incremento en los niveles de los marcadores NF, MBP, así como Olig-2 en los animales tratados con BDNF, en comparación con el grupo control (P<0.05).

Efecto del tratamiento con CTM-TA tras infarto subcortical. El tratamiento con CTM-TA mostró una buena recuperación funcional en comparación con el grupo control a los 28 días (P<0.05). El grupo sham no mostró déficit en ninguna de las evaluaciones. Respecto a la biodistribución de las CTM-TA administradas por vía i.v., no se observaron células en el

cerebro de los animales a las 24h, mostrándose marcaje positivo en órganos periféricos como el hígado, el pulmón y el bazo por inmunofluorescencia. Respecto al tamaño de lesión en RM, el tratamiento con CTM-TA estuvo asociado a una reducción del mismo a los 28 días en comparación con el grupo control (P<0.05). Además, dicha reducción en el grupo tratado con CTM-TA estaba asociada a un aumento de la conectividad cerebral en las imágenes de tractografía *in-vivo* (P<0.05), que era confirmada en los cortes histológicos mediante la técnica de tinción de mielina Cryomyelin (P<0.05). Respecto a la proliferación y muerte celular, el grupo de animales tratados con CTM-TA mostró un incremento en la proliferación celular medida por KI67 (P<0.05), así como un menor número de células TUNEL positivas (P<0.05) en comparación con el grupo control, a los 28 días. Dicho aumento de la proliferación celular correspondía a células que mostraban marcaje positivo neuronal (NeuN), glial (GFAP), así como de oligodendrocitos (Olig-2). Al analizar los niveles de marcadores asociados a reparación de sustancia blanca, se observó un incremento en los niveles de los marcadores NF, MBP, así como Olig-2 en los animales tratados con CTM-TA, en comparación con el grupo control a los 28 días (P<0.05). El estudio de proteómica por Orbitrap llevado a cabo sobre los sobrenadantes de las CTM-TA en cultivo permitió identificar más de 2.400 proteínas, entre ellas factores de crecimiento como el BDNF.

Conclusiones:

En un modelo experimental en ratas de infarto cerebral subcortical, observamos:

1. La administración del Factor Neurotrófico derivado del Cerebro (BDNF) mejora la recuperación funcional.

2. La administración del BDNF potencia mecanismos de reparación cerebral (aumento de células progenitoras de oligodendrocitos, incremento en la densidad de fibras de sustancia blanca y conectividad cerebral).

3. La administración de células troncales mesenquimales alogénicas derivadas de tejido adiposo (CTM-TA) mejora la recuperación funcional.

4. La administración de las CTM-TA potencia mecanismos de reparación cerebral (aumento de proliferación celular y de marcadores de sustancia blanca, incremento en la densidad de fibras de sustancia blanca y conectividad cerebral).

Los resultados de este trabajo sugieren que ambas estrategias terapéuticas (BDNF y CTM-TA) podrían ser útiles para la reparación del daño subcortical que acompaña a la lesión de pequeño vaso, así como para los infartos cerebrales de gran vaso en que resultan afectados tanto el cortex como el subcortex.

INTRODUCCIÓN

1. EL INFARTO CEREBRAL

La enfermedad cerebrovascular (ECV) comprende todo aquel trastorno en que uno o más vasos sanguíneos cerebrales se ven afectados por un proceso patológico, de tal modo que un área del encéfalo se ve alterada de forma transitoria o permanente por un proceso isquémico o hemorrágico [1]. Teniendo en cuenta la frecuencia del mismo, un 85% de los ictus son de tipo isquémico frente a un 15% que son hemorrágicos.

En el tipo isquémico, al obstruirse uno o más vasos sanguíneos cerebrales se produce una reducción del flujo sanguíneo cerebral (FSC) con la consiguiente entrada en isquemia de los tejidos adyacentes. De no revertirse el flujo, se produce la necrosis tisular y comienzan a aparecer los primeros déficit neurológicos. Dependiendo de la duración de la interrupción del flujo, tendrá lugar un infarto cerebral si es permanente, o un ataque isquémico transitorio (AIT). La gravedad vendrá siempre determinada por el tiempo de oclusión, la arteria afectada y la extensión del tejido afectado.

1.1. Epidemiología del infarto cerebral.

La incidencia y prevalencia de la ECV es alta y supone hasta un 10% del total de muertes en el mundo y uno de los principales motivos de hospitalización y de utilización de recursos sanitarios [2]. Según la OMS, el ictus representa la tercera causa de muerte y la primera de minusvalía en adultos en el mundo. En el caso concreto de España, es la segunda causa de muerte y la primera en la mujer [3], con una incidencia estimada de 187 casos por cada 100,000 habitantes al año [4]. Es además, la mayor causa de incapacidad, teniendo secuelas hasta en el 90% de los pacientes, que en el 30% los incapacitan para realizar sus actividades habituales [5].

En las últimas décadas el manejo de los pacientes ha sufrido importantes cambios e innovaciones clínicas como la implantación del código ictus, la atención en unidades de ictus especializadas, la trombolísis intravenosa y reperfusión precoz, el manejo de tratamientos preventivos y la rehabilitación. Sin embargo, a pesar de que las mejoras han

reducido de manera muy significativa la mortalidad y dependencia del infarto cerebral, todavía sigue siendo un importante problema de salud, consumiendo hasta el 4% del presupuesto sanitario global en España [6].

Por ello, el ictus continúa siendo un problema de salud de primera magnitud que obliga a identificar las mejores pautas de prevención y tratamiento para reducir en lo posible la incidencia del mismo, así como las dependencias de los pacientes.

2. PATOGENIA DEL INFARTO CEREBRAL

La isquemia cerebral se produce cuando el FSC se ve reducido a un nivel incapaz de mantener la homeostasis tisular, lo cual provoca alteraciones metabólicas y bioquímicas que alteran el funcionamiento del sistema nervioso, con la consiguiente manifestación de sintomatología clínica, y la muerte celular por necrosis o apoptosis si la situación no es revertida.

El FSC depende directamente del gradiente de presión de la sangre y de la resistencia vascular. En condiciones normales, el FSC es de 55-100mL/100g/min, lo que asegura un aporte continuo de oxigeno y glucosa para mantener los potenciales de membrana celular, así como la síntesis, liberación y recaptación de neurotransmisores. Cuando se produce una oclusión arterial, la reducción del FSC no es la misma en todo el territorio afectado. En este sentido, se distinguen diferentes regiones dentro de la zona afectada en función del FSC que reciban. Existe una zona central (núcleo) del infarto cerebral donde el FSC se encuentra por debajo de 10mL/100g/min, y en la que tiene lugar una rápida muerte celular [7], así como otra zona denominada "área de penumbra isquémica" o de "peri-infarto", en la que la perfusión cerebral se ve críticamente disminuida por debajo de 22mL/100g/min, pero en la que el consumo de oxígeno es todavía suficiente para preservar temporalmente la supervivencia tisular [8, 9]. Esta zona es en parte mantenida por la circulación colateral, y tiende a evolucionar a infarto cerebral si la reducción del FSC persiste y no es controlada adecuadamente [10, 11] (Figura 1).

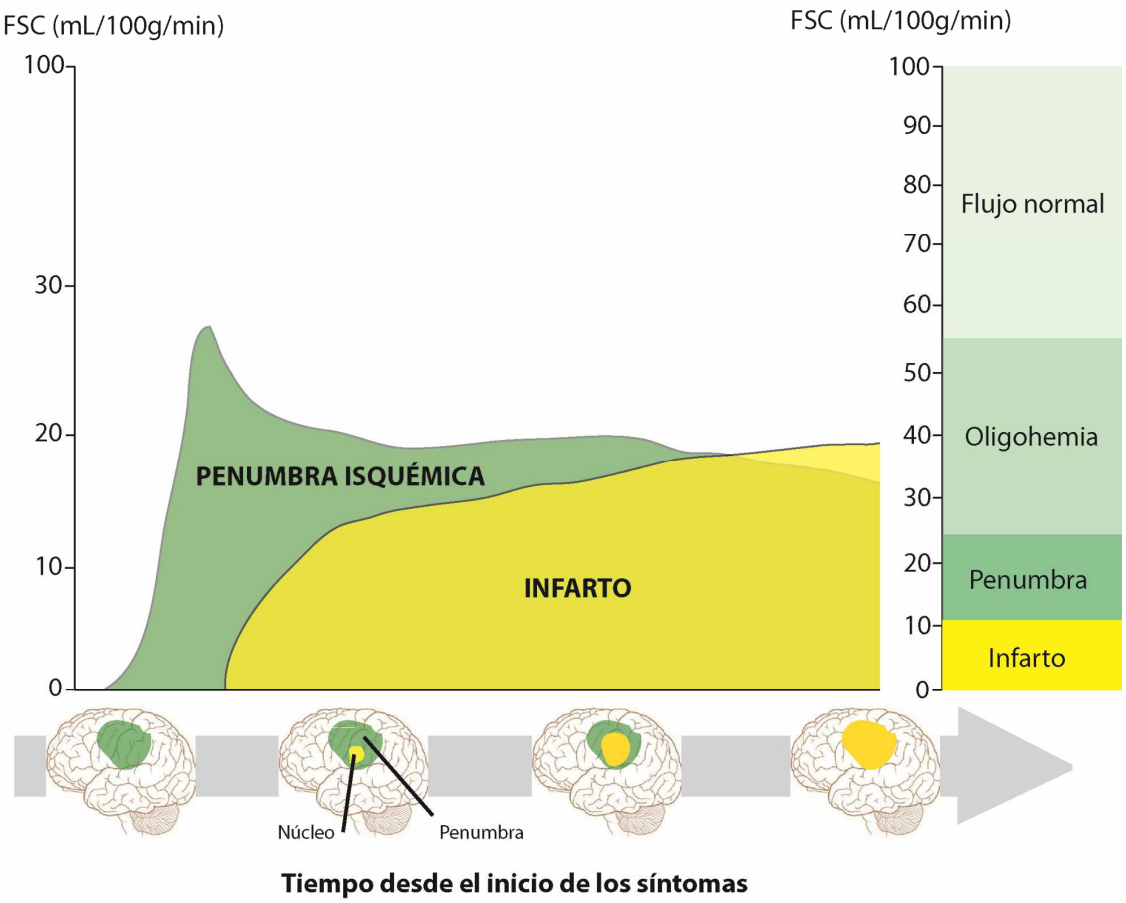

Figura 1. El flujo sanguíneo cerebral en la isquemia. *En condiciones normales, el flujo sanguíneo cerebral (FSC) se encuentra entre 55-100ml/100g/min, permitiendo el adecuado funcionamiento del metabolismo cerebral. La disminución del FSC por debajo de un determinado umbral origina alteraciones progresivas por las que la zona de penumbra o peri-infarto pasa a ser zona infartada. Dentro del territorio isquémico se distinguen diferentes zonas: núcleo del infarto (FSC<10ml/100g/min), zona de penumbra o peri-infarto (FSC<20ml/100g/min) y zona de oligohemia (25ml/100g/min<FSC<55ml/100g/min) (Tomada de: [12]).*

Además, esta zona de peri-infarto está sometida a una oleada de procesos metabólicos deletéreos propagados desde el núcleo isquémico como el estrés oxidativo y la respuesta inflamatoria entre otros, que conducen a la expansión del núcleo del infarto [13]. La progresión del daño celular en este *tejido en riesgo* hacia la irreversibilidad y la muerte es mediada por la puesta en marcha de la llamada "cascada isquémica", que constituye toda una secuencia de eventos que se suceden en el tiempo y desde etapas inmediatas a la reducción del FSC (Figura 2). Entre otros, incluyen la acidosis metabólica, la síntesis excesiva de óxido nítrico como mecanismo de compensación de la hipoxia, la liberación

descontrolada de neurotransmisores excitadores, la sobrecarga citosólica de calcio, el exceso de radicales libres, la peroxídación lipídica, así como una respuesta inflamatoria excesiva y la expresión de genes inductores de apoptosis e inflamación [14, 15].

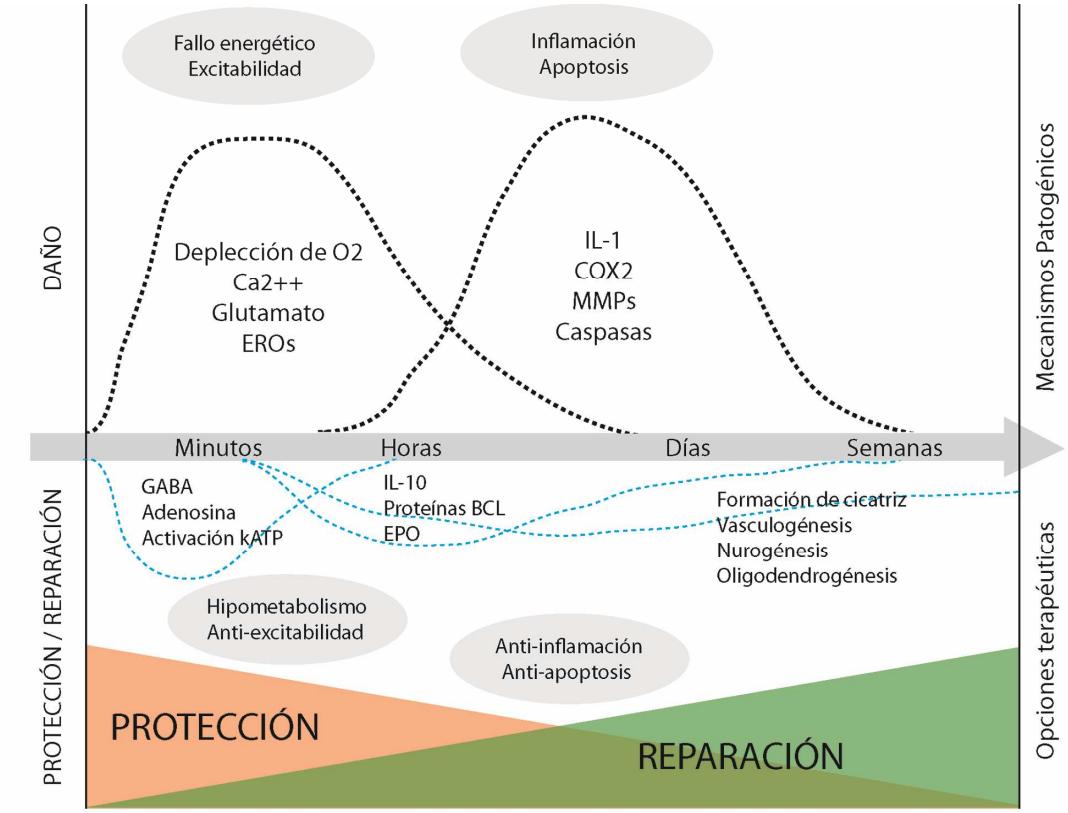

Figura 2. La cascada isquémica tras el infarto cerebral. *La liberación de mediadores de daño tras la lesión, pone en marcha la activación secuencial de mecanismos patogénicos como la inflamación y la apoptosis, que a su vez activan mecanismos de protección y reparación (anti-excitatorios, anti-inflamatorios, anti-apoptóticos) desde los primeros minutos hasta semanas después del infarto cerebral para evitar la progresión del daño y reparar la lesión. (Tomada de: [16])*

Aunque la mayor parte de los cambios tienen lugar en las etapas tempranas de la isquemia cerebral, otros vienen posteriormente, lo cual explica la progresión del daño durante horas e incluso días después de ocurrida la isquemia, aunque ésta no se agrave o a pesar de que haya reperfusión [17]. El periodo de tiempo durante el cual persiste el área de penumbra constituye una ventana potencial de oportunidad terapéutica que depende de la intensidad de la reducción del FSC, de la región cerebral afectada, así como de factores sistémicos como la glucemia, la presión arterial o la temperatura corporal [18], siendo por tanto un período de tiempo crucial durante el cual la restitución del FSC

regional y otras medidas protectoras pueden evitar la muerte de las células potencialmente viables [19]. Si este tiempo se sobrepasa, cualquier medida terapéutica no resultará beneficiosa. Tratándose de una enfermedad en la que el tiempo es un factor clave, cuanto más precoz sea la instauración del tratamiento mayor será la probabilidad de un efecto beneficioso. Esta idea es crucial para salvar la mayor cantidad de tejido en penumbra isquémica y en ese sentido, todavía hoy sólo pueden beneficiarse del rtPA un reducido porcentaje de pacientes, lo cual supone una importante limitación [20].

Por otro lado, hay que tener en cuenta que aunque las alteraciones ocurridas afectan a todos los tejidos comprometidos por la reducción del FSC, existe una vulnerabilidad celular selectiva frente al daño, de modo que algunas células y áreas cerebrales son más sensibles al daño que otras. Por ejemplo, las neuronas del hipocampo, núcleo amigdalino, corteza cerebral y cerebelo son más sensibles a la isquemia cerebral y sufren cambios estructurales más precozmente que las neuronas de otras localizaciones y que las células gliales [21]. Además, ha sido descrito cómo algunas estirpes celulares como los oligodendrocitos son especialmente vulnerables al daño isquémico, y a pesar de que el daño en los mismos y en las fibras de sustancia blanca es una observación frecuente en los pacientes de infarto cerebral, su estudio ha quedado relegado a un segundo plano en muchos trabajos [22, 23].

Como se ha mencionado anteriormente, dos de los mediadores de daño de la cascada isquémica son la inflamación y la muerte celular. En ese sentido, estudios previos de microarrays de expresión de mRNA en infarto cerebral cortical llevados a cabo por nuestro grupo, pudimos observar variaciones en genes asociados a ambos procesos [24].

La inflamación en el infarto cerebral se caracteriza por la acumulación de células inflamatorias y mediadores inflamatorios en el núcleo (core) de la lesión y en la zona de peri-infarto sobre los diversos componentes de la unidad neurovascular, formada por neuronas, glía y células endoteliales; junto con elementos sanguíneos circulantes, células presentadoras de antígenos perivasculares y astrocitos [25]. La respuesta inflamatoria

tiene un papel dual en el infarto cerebral. Mientras ésta se ha asociado a un aumento del daño cerebral y a un peor pronóstico, a la vez es fundamental para limpiar los elementos dañados y restos de debris celular, e iniciar así los procesos de neovascularización y remodelación tisular.

Respecto a la muerte celular, se ponen en marcha mecanismos que incluyen la necrosis y la apoptosis, que se pueden diferenciar por marcadores morfológicos y bioquímicos [26, 27]. La necrosis se caracteriza por el hinchamiento y lisis celular, induciendo progresivamente una respuesta inflamatoria [28]. Se trata de un fenómeno pasivo que depende de las alteraciones bioquímicas producidas por el déficit energético. Tras producirse cambios en la excitabilidad como consecuencia de la reducción del FSC, se desencadena el incremento citoplasmático de calcio, la activación secundaria de enzimas líticas que destruyen componentes celulares (como es el exceso del NO), así como un incremento de la respuesta inflamatoria y exceso de radicales libres que tendrán un papel fundamental durante el daño por reperfusión [14, 15, 29]. Por el contrario, en la apoptosis o muerte celular programada, el proceso es activo y se requiere un consumo de ATP. Este mecanismo afecta mayoritariamente al área de penumbra y en las células tiene lugar la fragmentación del ADN, la condensación de la cromatina y la ruptura citoplasmástica en cuerpos apoptóticos, que son fagocitados impidiendo la liberación del contenido citoplasmático y, por tanto, una respuesta inflamatoria exacerbada.

Pese a que en los últimos años la mayoría de los estudios de infarto cerebral en modelos animales experimentales se han centrado en el daño cortical, la clínica humana es muy distinta y los infartos cerebrales muestran afectación subcortical con daño en las fibras de sustancia blanca (axón y mielina) (Figura 3). De hecho, mientras que en roedores el volumen de sustancia blanca no llega a superar el 15% del volumen cerebral total, en los pacientes hasta el 50% del volumen está ocupado por fibras, por lo que la relevancia del estudio de los mecanismos de daño así como de reparación de las mismas en las lesiones subcorticales es fundamental [23]. En el pasado, la mayoría de las terapias protectoras

iban dirigidas a la superviviencia de las neuronas [30], sin embargo, hoy en día es sabido que como consecuencia del infarto cerebral, el conjunto de la integridad de la unidad neurovascular queda comprometida, y es por tanto diana de actuación [25, 31, 32].

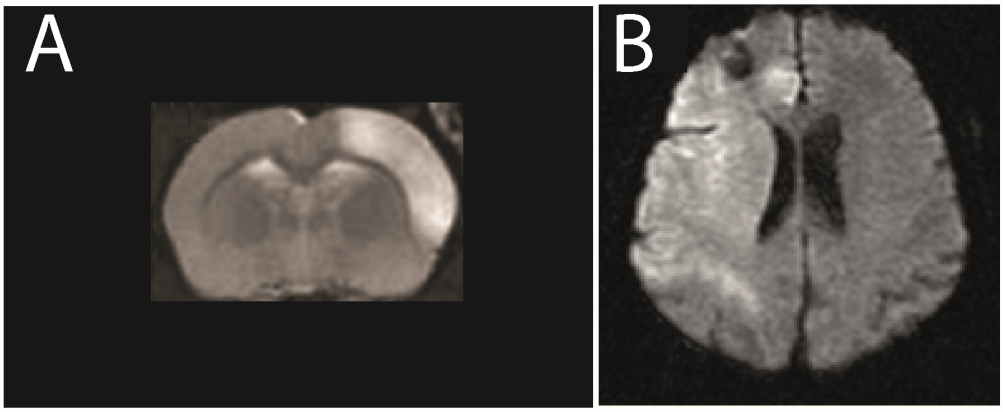

Figura 3. Imágenes de resonancia magnética (T2) tras infarto cerebral en modelo animal experimental y en paciente. *(A) Lesión de infarto cerebral en el modelo de oclusión permanente de la arteria cerebral media en rata, donde puede observarse afectación de tipo cortical. (B) Lesión de infarto cerebral en el territorio de la arteria cerebral media (ACM) en un paciente donde puede observarse afectación cortico-subcortical.*

Tras la isquemia cerebral, algunos marcadores estructurales como la proteína básica de mielina (MBP), o el marcador de filamentos intermedios Neurofilamento (NF) disminuyen en el parénquima cerebral [33]. Además, hoy sabemos que uno de las estirpes celulares más vulnerables al daño isquémico son los oligodendrocitos [34]. En este sentido, el factor de transcripción de oligodendrocitos (Olig-2), juega un importante papel, y se ha podido observar una disminución en sus niveles a las pocas horas del infarto cerebral. Del mismo modo, tras el daño también disminuyen los niveles de marcadores de células precursoras de oligodendrocitos (CPO), como las proteínas 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase), Adenomatous polyposis coli (APC/CC1) o el receptor del factor de crecimiento derivado de plaquetas (PDGFR) [24].

2.1 Plasticidad, protección y reparación cerebral.

Paralelamente al daño, se ponen en marcha mecanismos de protección y reparación cerebral que son activados como un continuo en el contexto de la plasticidad cerebral [16] [24]. Estos incluyen procesos como la neurogénesis, oligodendrogénesis, sinaptogénesis, angiogénesis, regeneración axonal y remielinización, los cuales contribuyen a la reparación de la unidad neurovascular y son modulados en un contínuo y de forma sincronizada. Así, ya a los pocos días se activan procesos de proliferación celular y neurogénesis desde la zona subventricular (ZSV) y el giro dentado del hipocampo, así como la modulación de la expresión de genes asociados al desarrollo del sistema nervioso central (SNC). En modelos animales experimentales, ha podido demostrarse cómo existe un pico de proliferación a los 7 días en nichos neurogénicos como la ZSV cuya actividad puede ser potenciada con fines terapéuticos [35]. Hoy sabemos que estas células proliferantes son células progenitoras neurales con capacidad de diferenciación a distintos linajes como neuroblastos o CPO. Desde los nichos neurogénicos, la población de neuroblastos es reclutada hacia las áreas que bordean el infarto, pudiendo así diferenciarse a neuronas adultas [36]. Este proceso va asociado a la formación de nueva vasculatura y al remodelado de vasos sanguíneos después del infarto cerebral [37], así como sirven de estímulo neurogénico [38] a través de diferentes factores que son modulados desde fases tempranas, como el factor de crecimiento del endotelio vascular (VEGF)[39], o el factor neurotrófico derivado del cerebro (BDNF) [24]. Posteriormente se activan procesos tardíos como la remielinización, que necesita de la presencia de células adultas y conexiones neuronales funcionales para poder ser inducida de manera eficaz [40].

Al mismo tiempo, otros factores participan en los mecanismos de plasticidad cerebral de forma inhibitoria, como es el caso de las proteínas Lingo1 y Nogo-A, las cuales intervienen en el proceso de remodelado tisular impidiendo, o bien la remielinización mediante la proteína Lingo1 [41], o bien la formación de nuevas conexiones neuronales

mediante la proteína NogoA [42]. El papel, no obstante, de los niveles de las proteínas Lingo1 y Nogo-A en cerebro y su posible papel como marcador de plasticidad todavía no han sido estudiados en profundidad.

3. MODELOS ANIMALES DE INFARTO CEREBRAL.

La experimentación animal es fundamental para poder conocer los fenómenos patogénicos y fisiopatológicos que acontecen durante la isquemia cerebral, así como para poder probar estrategias terapéuticas antes de ser ensayadas en humanos. En líneas generales, los modelos animales deben cumplir una serie de condiciones para ser considerados de utilidad. En primer lugar, deben ser factibles y sencillos. Deben emular la clínica humana y al mismo tiempo ser fácilmente reproducibles y tener escasa variabilidad inter e intra observador. Existen múltiples modelos experimentales de infarto cerebral, y aunque se han utilizado distintos animales, el más habitual es la rata. Ello es debido a su fácil manejo y monitorización fisiológica, su pequeño tamaño, su grado de homología genética [43] y la similitud entre la anatomía y fisiología de la vasculatura cerebral con los humanos [44]. Sin embargo, es importante recordar que aunque estos reflejen elementos de la patología humana, los modelos animales son siempre aproximaciones que nunca reproducen la enfermedad. Estas discrepancias entre los modelos animales y los estudios en pacientes enfatiza la importancia de realizar estudios en distintos tipos de modelos animales que reflejen las distintas características que observamos en los pacientes, con el fin de mejorar entre todos ellos la traslación de los resultados a la clínica [45].

Los modelos animales más utilizados en el infarto cerebral son el de ligadura de la arteria cerebral media (ACM) tras craneotomía, el de oclusión intraluminal de ACM mediante la introducción de un filamento desde la arteria carótida interna (ACI), el modelo de oclusión por embolia con coágulo de sangre autóloga y el modelo de trombosis intravascular inducida fotoquímicamente. En todos ellos se ha ido produciendo un cierto

grado de perfeccionamiento con el fin de reducir la mortalidad, así como la variabilidad en el tamaño de infarto [46-50].

La mayoría de modelos experimentales son de afectación cortical, como es el caso del modelo de ligadura de ACM así como el de trombosis inducida fotoquímicamente, o bien son modelos de daño masivo cortico-subcortical como son el modelo de oclusión por coágulo de sangre autóloga o el de oclusión intraluminal de la ACM. Sin embargo, los mecanismos de daño y reparación de las fibras de sustancia blanca subcortical han sido menos estudiados, motivo por el cual serán el objeto de estudio de este trabajo de Tesis Doctoral, tal y como se presenta a continuación. Así, en los últimos años se han puesto a punto nuevos modelos animales que reproducen la afectación sobre las fibras subcorticales, como es el caso de los modelos de daño en sustancia blanca a nivel del cuerpo calloso [51, 52]. Aunque estos modelos reproducen fielmente la afectación sobre las fibras nerviosas y permiten el estudio de los mecanismos de daño y formación de nuevas fibras, no se produce déficit funcional en los animales cuando dichas fibras son lesionadas, lo cual supone una importante limitación desde un punto de vista traslacional. En los pacientes las lesiones subcorticales conllevan habitualmente déficit funcional, siendo las más relevantes las que acontecen sobre las fibras de proyección que conectan la corteza cerebral con los centros inferiores como la cápsula interna. De hecho, infartos de relativamente pequeño tamaño sobre estas fibras pueden conllevar importantes déficits neurológicos. Además de verse afectadas la atención, la velocidad de procesamiento de la información, así como la consciencia, las lesiones en dichas fibras pueden dar lugar a fallos de tipo motor. Debido a la perspectiva translacional del grupo de investigación, con el fin de obtener un modelo animal con afectación de las fibras de proyección subcorticales, se puso a punto el modelo de infarto cerebral subcortical por inyección de Endotelina (ET)-tipo 1, modelo en el que las fibras de proyección son lesionadas a este nivel en la rata (Figura 4).

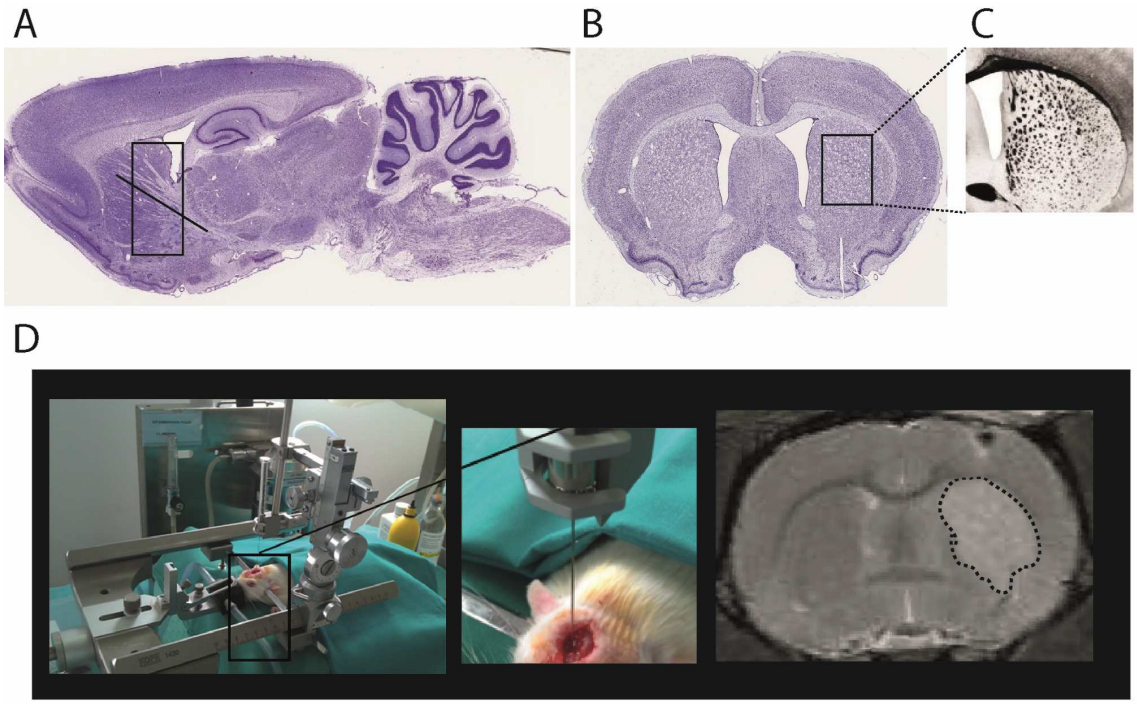

Figura 4. Distribución de la sustancia blanca subcortical en el cerebro de la rata y el modelo de infarto subcortical por inyección de Endotelina-1 *(**A**) Tinción de Nissl sobre corte longitudinal. Pueden observarse al detalle (recuadro negro) las fibras de proyección ascendentes y descendentes que conectan la corteza cerebral y los centros inferiores cerebrales. Una línea negra indica el eje principal sobre el que se encuentran distribuidas las fibras de proyección en su mayoría. (**B**) Tinción de Nissl sobre corte coronal. En el recuadro se detallan las fibras de proyección que son dañadas en el modelo animal de infarto subcortical por inyección de Endotelina tipo 1 (ET-1) (**C**) Reconstrucción de corte coronal en tinción de Cryomyelin para fibras de mielina. (**D**) Inducción del infarto cerebral subcortical por inyección de Endotelina (ET) tipo 1 en rata. A la izquierda puede observarse la disposición de los animales colocados en el marco estereotáxico para la inyección intracerebral de ET-1 (detalle en la fotografía central). Los animales quedan expuestos a anestesia inhalatoria durante todo el proceso quirúrgico. Tras hacer una pequeña incisión y craneotomía, se lleva a cabo la inyección de ET-1 (1uL; 0.25 µg/µL) a nivel subcortical siguiendo coordenadas estereotáxicas (+0.4 mm anteroposterior, +3.5 mm lateral, +6 mm dorsoventral) respecto a la sutura bregmática, a una velocidad de 0,2uL/min. A la derecha se muestra una imágen representativa de resonancia magnética (T2) adquirida a día 1 tras la cirugía donde puede visualizarse el área de lesión.*

Imágenes de Nissl tomadas del Atlas de acceso libre BrainMaps – [www.brainmaps.org]. Imagenes de Cryomyelin, resonancia magnética e imágenes del procedimiento quirúrgico obtenidas en el laboratorio de Neurociencia y Cerebrovascular del Hospital Universitario La Paz.

Con el objetivo de obtener infartos reproducibles, a lo largo del estudio se optimizaron la dosis y velocidad de inyección de ET-1 adecuadas, para lo cual nos basamos en estudios previos [53]. Tal y como se describirá en los capítulos posteriores, la reproducibilidad de

los infartos, así como la homogeneidad del tamaño de lesión pueden ser comprobados mediante la adquisición de imágenes en T2 de resonancia magnética a las 24h tras la cirugía (Figura 4D). Aunque la complejidad de las fibras de proyección es menor en la rata en comparación con los pacientes, puede observarse déficit funcional motor cuando éstas son lesionadas. Dicho déficit puede ser evaluado mediante tests funcionales que evalúan el comportamiento motor en los animales, como el de la barra longitudinal [54], el test del cilindro rotatorio [55] o el de Rogers [56]. Por este motivo, el modelo de infarto subcortical por inyección de ET-1 es un modelo de alto interés translacional para la búsqueda de nuevas estrategias terapéuticas.

4. LOS FACTORES TRÓFICOS Y LA TERAPIA CELULAR COMO ESTRATEGIAS PARA LA ESTIMULACIÓN DE LA REPARACIÓN CEREBRAL

Lejos de ser un proceso sencillo, la recuperación del infarto cerebral es un proceso complejo y dinámico que incluye respuestas frente al daño en las zonas de lesión, así como procesos de reparación activos en los que está implicado el órgano en su conjunto y que deben ser debidamente sincronizados para la correcta remodelación tisular. En este sentido, en los últimos años ha quedado demostrado cómo los procesos implicados en la reparación cerebral pueden ser estimulados mediante la administración de factores tróficos, así como mediante la terapia celular.

4.1. Los factores tróficos en el infarto cerebral en modelos animales

Tras el descubrimiento del factor de crecimiento nervioso (del inglés NGF) en los años 50's por Levi-Montalcini y Hamburguer [30], se abrió una nueva era en el campo de la fisiología en la que los factores de crecimiento emergían como nuevas moléculas capaces de promover la reparación de tejidos y por tanto, eran una nueva estrategia terapéutica potencial en los pacientes. A partir de este descubrimiento el número de moléculas capaces de potenciar la reparación cerebral ha ido en constante aumento. En esencia, los

factores tróficos (también llamados factores de crecimiento) son moléculas que desempeñan funciones sobre la señalización celular, siendo capaces de estimular, entre otras, la progresión del ciclo celular y por tanto, la formación de nuevas células en el cerebro adulto. En ese sentido, estudios en animales de infarto cerebral han mostrado cómo la administración de factores tróficos como el factor de crecimiento básico de fibroblastos (del inglés bFGF), son capaces de estimular procesos de neurogénesis y promover la proliferación de células progenitoras, tanto en el giro dentado del hipocampo como la ZSV [35, 57]. Otros factores tróficos, como el VEGF, participan en la estimulación de la angiogénesis y su administración tras infarto cerebral promueve la recuperación funcional en modelos animales [58-60]. Del mismo modo, el factor de crecimiento de insulina (del inglés IGF), ejerce un papel importante sobre la inhibición de la apoptosis y la estimulación de la proliferación celular [61, 62]. Tal y como se ha mencionado, existen múltiples factores tróficos con capacidad para potenciar la plasticidad cerebral tras infarto cerebral. Dados los efectos pleiotrópicos de los mismos sobre múltiples dianas celulares, es necesario estudiar en detalle sus mecanismos de acción en los modelos animales.

Uno de los factores que más interés ha suscitado en los últimos años en el infarto cerebral es el BDNF, el cual actúa de forma específica y con gran afinidad por los receptores de kinasas tipo B (TRKB), activando rutas de transducción de señales que promueven la activación de kinasas asociadas a estímulos mitógenos extracelulares, y que a su vez previenen la muerte neuronal tras la isquemia. Esta potenciación de la supervivencia neuronal se manifiesta en una modulación de los niveles intracelulares de las proteínas reguladores de la apoptosis Bax/Bcl-2 [63]. Estudios previos han mostrado cómo la administración intravenosa de este factor tras infarto cerebral cortical, es capaz de promover la migración de progenitores desde la ZSV, así como el aumento de marcadores implicados en neurogénesis, como la proteína doblecortina (DCX) y el antígeno neuronal NeuN [64]. Sin embargo, el efecto que podría tener la administración terapéutica de dicho factor sobre la estimulación de la oligodendrogénesis y la reparación

de las fibras nerviosas de sustancia blanca todavía no ha sido estudiado. En este sentido se ha sugerido cómo el BDNF podría ser un factor trófico especialmente interesante, ya que las propias células gliales de las fibras de sustancia blanca lo producen con fines protectores y reparadores [65]. De este modo, parece factible que su administración sistémica tras el daño cerebral pudiera potenciar dicho efecto y por ello sería de alto interés investigar el efecto terapéutico del BDNF sobre la reparación de las fibras nerviosas en modelos animales experimentales de infarto cerebral subcortical.

4.2. La terapia celular en el infarto cerebral en modelos animales

En modelos animales experimentales de infarto cerebral, la administración de células troncales neurales (CTN) es capaz de promover la regeneración axonal, la neurogénesis, la gliagénesis, así como la activación de genes asociados a factores de crecimiento [66-69]. En algunos estudios los autores han observado además, una reducción en el volumen de infarto tras el tratamiento, así como una reducción en la muerte celular [70]. Estos buenos resultados se observan con otras estirpes celulares, como las células derivadas del cordón umbilical, o las células troncales mesenquimales (CTM), bien sean derivadas de médula ósea (CTM-MO) o tejido adiposo (CTM-TA) [55, 56, 71-73]. De hecho, el descubrimiento en el año 2001 de la existencia de CTM en el tejido adiposo en adultos [74], ha sido una revolución, ya que supone una fuente inagotable de células en adultos fácilmente disponible tras las liposucciones, con la ventaja añadida de no presentar conflictos de tipo ético [75, 76]. Desde entonces, los efectos beneficiosos del tratamiento con CTM-MO o CTM-TA en el infarto cerebral han sido ampliamente descritos en la literatura y numerosos estudios han mostrado buenos resultados en ese sentido, siendo las CTM-TA de especial interés dada su especial abundancia y fácil obtención. Aunque los mecanismos de acción de las CTM no se conocen, hoy sabemos que la inmunomodulación, así como la liberación de sustancias capaces de promover la recuperación funcional a través de la

modulación de los mecanismos de protección y reparación cerebral como la neurogénesis, angiogéneis, así como la oligodendrogénesis parecen jugar un papel importante [75-79].

Con el fin de optimizar la terapia celular en el infarto cerebral, todavía quedan algunas cuestiones prácticas importantes sin resolver, como son: cuál sería la dosis apropiada, cuál sería la procedencia de las células y el tipo celular adecuado, así como cuál sería el tiempo y vía de de administración oportunas para su futura traslación a pacientes.

En cuanto a las dosis, distintas cantidades se han administrado en los diferentes estudios [80, 81]. En modelos animales, la dosis de $2*10^6$ CTM-TA/kg administradas por vía intravenosa ha dado buenos resultados en estudios independientes [55, 82, 83]. Aunque estudios posteriores deberían confirmar si el uso de dosis repetidas podrían aumentar el efecto terapéutico [84].

En cuanto al tiempo de administración, sabemos que la administración en fase aguda es crucial con el fin de potenciar los efectos protectores y reparadores ya desde estadios tempranos [75]. En modelos animales, la administración de CTM-TA a las 24h del infarto cerebral ha dado buenos resultados [55, 82, 83].

Respecto a la procedencia celular, las células pueden ser xenogénicas (proceden de otra especie), autólogas (del mismo individuo) y alogénicas (procedentes de diferente individuo, pero de la misma especie). Aunque la procedencia óptima sería la autóloga, su principal limitación es que sólo sería posible la administración varias semanas después del infarto debido al tiempo necesario para la expansión de las células, haciendo imposible la administración en fase aguda. Para solventar este problema tenemos la administración alogénica, la cual ha demostrado eficacia en modelos animales experimentales de infarto cerebral, no observándose fenómenos de rechazo [55, 56, 78, 79, 85, 86]. En ese sentido, las CTM no expresan antígenos del complejo mayor de histocompatibilidad (CMH) de clase II, así como carecen de antígenos del complejo de histocompatibilidad (HLA) y de las moléculas coestimuladoras CD40, CD80 y CD86 [87-89]. A su vez, el hecho de que

presenten bajos niveles de expresión de antígenos CMH de clase I, permite la no activación de células T ni de respuestas inmunológicas de tipo secundario [89].

Con respecto a la ruta de administración, hace años se apoyaba la idea de que era necesaria la implantación de las células en el cerebro para promover la recuperación funcional. Por este motivo, la mayoría de estudios se centraban en rutas de administración invasivas como la intracerebral [77, 85, 90]. Sin embargo, otras vías menos invasivas también han mostrado resultados prometedores como la intracarotidea (i.c.) [78, 91, 92], intravenosa (i.v.) [56, 77] e intranasal [93], siendo más apropiadas para su traslación a la clínica.

Respecto a la estirpe celular, diferentes líneas celulares se han empleado en modelos animales de infarto cerebral con buenos resultados [75, 77, 82, 94-96]. De entre todas ellas, nos centraremos en las células CTM-TA, las cuales presentan numerosas propiedades que las hacen especialmente interesantes como son su abundancia [97], su alta capacidad de autorrenovación, su capacidad de diferenciación a diferentes linajes celulares como osteoblastos, condrocitos, adipocitos, neuronas y glía [98], así como su capacidad para potenciar la reparación de tejidos en estudios *in vitro* e *in vivo* [99]. Además, un estudio comparativo reciente de nuestro grupo de investigación mostró cómo las CTM-TA eran igual de eficaces que las CTM-MO sobre la recuperación funcional y la estimulación de los procesos implicados en la plasticidad cerebral tras infarto cerebral cortical en un modelo animal experimental en rata [55].

En definitiva, aunque existen numerosas evidencias de los efectos beneficiosos de la administración de células troncales en los modelos animales de infarto cerebral, los procesos que subyacen a esa buena recuperación funcional son aún desconocidos. En diferentes estudios independientes se ha demostrado cómo las CTM promueven la recuperación funcional tras infarto cerebral [72, 100], reduciendo el volumen de lesión, y la inflamación [73]. Sin embargo, a día de hoy todavía desconocemos si las CTM-TA pueden ejercer un efecto terapéutico en infartos con afectación subcortical al inducir la

estimulación de procesos como la oligodendrogénesis y la reparación de las fibras nerviosas de sustancia blanca. En este sentido, trabajos recientes sugieren cómo la administración intravenosa de CTM-TA tras infarto cerebral cortical promueve un aumento de los niveles de marcadores asociados a reparación en los animales tratados [55]. Así, sería de alto interés estudiar el efecto terapéutico de la administración de CTM-TA en modelos animales de infarto cerebral subcortical, con especial atención a su posible efecto sobre la estimulación de la reparación de las fibras nerviosas de axón y mielina.

HIPÓTESIS Y OBJETIVOS

A la vista de los antecedentes previos, nos planteamos la siguiente hipótesis:

Hipótesis:

Los mecanismos de reparación en el infarto cerebral cortical pueden ser potenciados mediante la administración de factores tróficos, así como mediante la terapia celular. Sin embargo, todavía no ha sido suficientemente demostrado si dichas terapias son capaces de potenciar la reparación de las fibras de sustancia blanca tras infarto cerebral subcortical. En este sentido, es posible que la administración del factor neurotrófico derivado del cerebro (BDNF) así como, la administración de células troncales mesenquimales derivadas de tejido adiposo (CTM-TA) tras infarto subcortical pueda ser capaz de potenciar la reparación de las fibras de sustancia blanca, mejorando la conectividad cerebral y la recuperación funcional.

Objetivos:

En un modelo experimental en rata de infarto cerebral subcortical con afectación de sustancia blanca pretendemos:

1. Analizar el efecto de la administración intravenosa del Factor Neurotrófico derivado del cerebro (BDNF) sobre la recuperación funcional.
2. Estudiar el efecto de la administración intravenosa de BDNF sobre la reparación de las lesiones de sustancia blanca, la proliferación celular, crecimiento de fibras y recuperación de la conectividad cerebral.
3. Analizar el efecto de la administración intravenosa de células troncales mesenquimales derivadas de tejido adiposo (CTM-TA) sobre la recuperación funcional.
4. Estudiar el efecto de la administración intravenosa de CTM-TA sobre la proliferación y diferenciación de células precursoras de oligodendrocitos (CPO), la reparación de las lesiones de sustancia blanca, el crecimiento axonal, así como la recuperación de la conectividad cerebral.

El presente trabajo de Tesis Doctoral ha quedado recogido en tres artículos que constituyen cada uno de los capítulos que se presentan a continuación. El primero, publicado en *Journal of Cellular and Molecular Medicine*, constituye un trabajo de revisión en el que se expone el potencial de los factores tróficos y la terapia celular como estrategias para potenciar la reparación cerebral tras el ictus, tanto en modelos animales experimentales como en pacientes. En el segundo trabajo, publicado en *Stroke*, se demuestra el potencial terapéutico de la administración intravenosa del factor neurotrófico derivado del cerebro (BDNF en inglés) tras infarto cerebral subcortical en ratas y en particular su efecto sobre la estimulación de la oligodendrogénesis y la mejora en la conectividad cerebral, así como la reparación de las fibras de sustancia blanca. El tercer trabajo, publicado en la revista *Stem Cell Research and Therapy*, muestra el potencial terapéutico de la administración intravenosa y alogénica de células troncales mesenquimales derivadas de tejido adiposo (CTM-TA) tras infarto cerebral subcortical, en el que se observa una mejoría funcional asociada a una estimulación de la proliferación celular, un aumento de la conectividad cerebral, así como un incremento en los niveles de marcadores de reparación de las fibras de sustancia blanca.

Artículo número 1:

TROPHIC FACTORS AND CELL THERAPY TO STIMULATE BRAIN REPAIR AFTER ISCHAEMIC STROKE

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Trophic factors and cell therapy to stimulate brain repair after ischaemic stroke

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  - **Trophic factors**
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- **Stem cell therapies**
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Abstract

Brain repair involves a compendium of natural mechanisms that are activated following stroke. From a therapeutic viewpoint, reparative therapies that encourage cerebral plasticity are needed. In the last years, it has been demonstrated that modulatory treatments for brain repair such as trophic factor- and stem cell-based therapies can promote neurogenesis, gliogenesis, oligodendrogenesis, synaptogenesis and angiogenesis, all of which having a beneficial impact on infarct volume, cell death and, finally, and most importantly, on the functional recovery. However, even when promising results have been obtained in a wide range of experimental animal models and conditions these preliminary results have not yet demonstrated their clinical efficacy. Here, we focus on brain repair modulatory treatments for ischaemic stroke, that use trophic factors, drugs with trophic effects and stem cell therapy. Important and still unanswered questions for translational research ranging from experimental animal models to recent and ongoing clinical trials are reviewed here.

Keywords: brain plasticity • brain protection • brain repair • trophic factors • stem cell therapy

Brain repair after ischaemic stroke

Protective therapies that focused on saving just the neural cells instead of protecting all the components of the neurovascular unit have consistently failed [1]. Rather than being simple, recovery from ischaemia is a complex and highly dynamic process that includes not only injury and response signals within the lesions but also active self-repair processes that occur in the whole organ [2–5] and that should be precisely synchronized for tissue remodelling. Neurogenesis, gliogenesis, oligodendrogenesis, synaptogenesis and angiogenesis are brain repair-associated processes that are activated following stroke. In recent decades, animal models of cerebral ischaemia and clinical research have demonstrated how brain repair processes can be actively modulated by the administration of both trophic factors and stem cells.

We should first consider that protection and repair mechanisms are activated and work together from the very beginning of cerebral ischaemia (Fig. 1). The accompanying hypoxia and glucose

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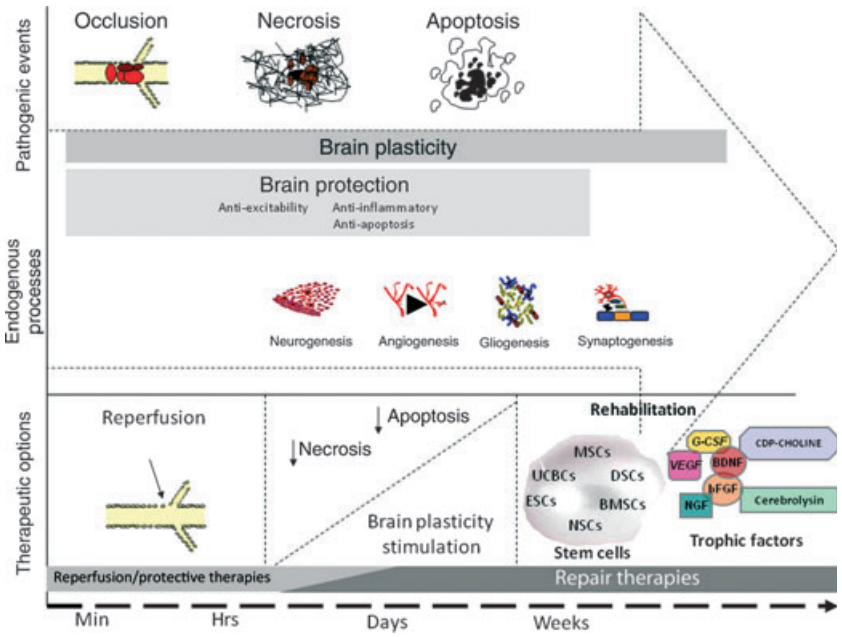

Fig. 1 Pathogenic mechanisms and therapeutic options in cerebral infarct. Time line for the mechanisms and therapy involved in endogenous protection and brain protection-repair after ischaemic stroke. MSCs: mesenchymal stem cells; UCBCs: umbilical cord blood cells; DSCs: dental stem cells; ESCs: embryonic stem cells; BMSCs: bone marrow stem cells; NSCs: neural stem cells; G-CSF: granulocyte colony-stimulating factor; VEGF: vascular endothelial growth factor; BDNF: brain-derived neurotrophic factor; NGF: nerve growth factor; bFGF: basic fibroblast growth factor; IGF-1: insulin growth factor-1; EPO: erythropoietin.

deprivation, cell death programs and immunological events of ischaemia are initiated to remove damaged cells and tissue debris and to prepare injured areas for repair processes [6–8] and as a response to injury, transcriptional programs associated with axonal sprouting, survival and myelin formation are activated and maintained from the very beginning [9, 10]. Research is now focused on how to modulate these processes to preserve all the structures that make up the neurovascular unit, including microvessels and pericytes (vascular protection), neurons and their axons (neuroprotection), astrocytes and other supportive cells such as oligodendroglia [11].

Synchronized events after damage may allow initial deleterious signals to transition into beneficial effects and recovery [12]. During the early acute phase, blood-brain barrier disturbances predominate and matrix proteases like MMP-4 or MMP-9 are essential for neurovascular remodelling, while during the late phase, other processes, such as angiogenesis, may provide the critical substrate for remodelling. Understanding how neurovascular signals and substrates make the transition from initial injury to angiogenic recovery is important for obtaining new therapeutic options as a cerebral infarct is a highly complex condition whose effects might extend beyond time (time since the ischaemic insult) and location (communication between brain ischaemic regions and healthy areas).

Trophic factors, stem cell therapy and rehabilitation have all been shown to exert potential therapeutic effects by modulating brain repair- associated mechanisms (Fig. 2). In experimental animals, increased levels of neurogenesis, gliogenesis, oligodendrogenesis and angiogenesis accompanied with better functional recovery have been widely reported after treatment [13, 14]. Such promising preclinical results have led to multiple clinical trials in the last years. In this review, we will discuss recent reports from both pre-clinical and clinical studies that raise important new questions and concerns for further advances in the field.

Trophic factor-based therapies

The discovery of nerve growth factor (NGF) in the 1950s by Levi-Montalcini and Hamburger [15] opened a promising new era in physiology in which the growth-factor induced regeneration of damaged tissues seemed to be possible and its therapeutic potential has been explored in both experimental animals and clinical trials (Table 1). As could expected, new hopes and fundamental questions have emerged over the last years. This approach could be based on the direct administration of trophic factors, and of drugs with trophic effects.

Experimental animal models

Trophic factors. The reported number of biological modulatory molecules that mediate in brain repair is high and ever-growing. Besides NGF, which has been reported to improve cholinergic function, stimulate axonal growth, cerebral perfusion and neurogenesis by stimulating proliferation through tyrosine kinase receptor signalling [16, 17], the administration of other factors, like basic fibroblast growth factor (bFGF), has been shown to promote neurogenesis in both intact and ischaemic brain [18]. Indeed, intracysternal administration 1 day after experimental stroke in rats has been shown to stimulate progenitor cell proliferation in the subventricular zone (SVZ) and dentate gyrus (DG), important areas for the development of new neurons in the adult brain [19]. While higher levels seem to be required after damage [9], it is important to emphasize that trophic factors not only act in disease but also under normal conditions to

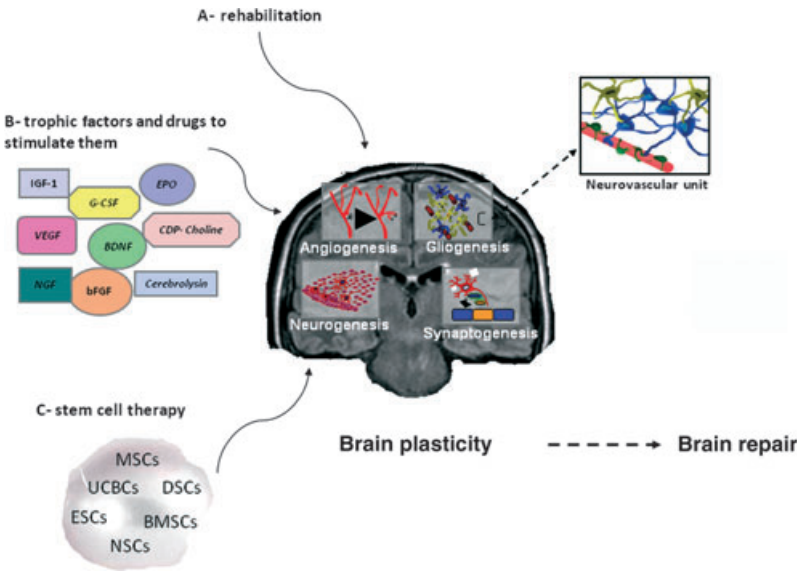

Fig. 2 Brain repair therapies through brain plasticity enhancement. Mechanisms underlying cerebral plasticity associated with repair processes. G-CSF: granulocyte colony-stimulating factor; VEGF: vascular endothelial growth factor; BDNF: brain-derived neurotrophic factor; NGF: nerve growth factor; bFGF: basic fibroblast growth factor; IGF-1: insulin growth factor-1; EPO: erythropoietin; MSC: mesenchymal stem cells; UCBC: umbilical cord blood cells; DSC: dental stem cells; ESC: embryonic stem cells; BMSC: bone marrow stem cells; NSC: neural stem cells.

maintain tissue homeostasis. This has been reported in brain-derived neurotrophic factor (BDNF) signalling, impairment of which may cause progressive neuronal dysfunction in animal models [20]. In this sense, intravenous administration of BDNF during the 5 days following cortical photothrombotic stroke is associated with enhanced migration of progenitor cells from the SVZ and increased neurogenesis in the DG on DCX- and NeuN-stained slices [21].

How can brain repair be modulated by the action of factors like BDNF? Although still unclear, white matter glial cells have been reported to play a key role in protecting and promoting the regeneration of nerve fibres by producing BDNF itself [22]. Also, prostacyclin, an important hormone released in response to vascular damage is stimulated around cerebral arteries when this factor is present [23]. From a genetic perspective, it is known that BDNF can activate NF-kB through the TrkB-PI3-Kinase-Akt pathway [24] and that this activation leads to the downstream activation of genetic programs that contribute to protecting cells from stress conditions such as serum starvation, glutamate toxicity or ischaemia [25], all of which occur at the beginning of the ischaemic insult.

It bears mentioning that trophic factors not only enhance single processes like neurogenesis, but they also exert pleiotropic effects on other biological pathways such as vascular function, immune cell function or cell death. In this sense, it was recently reported that the preserved neuronal loss and reduced number of TUNEL-positive cells after intranasal administration of BDNF might also be due to modulation of local inflammation by this factor, which would reduce tumour necrosis factor-α (TNF-α) levels and augment those of interleukin (IL)-10 [26].

However, in addition to all of this pleiotropic interplay, the activity of most of these factors within the brain under ischaemic conditions is not clear. After the hypoxic insult, many hypoxia-response genes such as HIF-1alpha are upregulated, triggering downstream changes in other interacting genes such as vascular endothelial growth factor (VEGF), which is the key gene for the angiogenesis induced in penumbral regions of the brain. This angiogenesis is known to depend on several factors including VEGF, VEGFR2, Angiopoietins 1 and 2 and its Tie2 receptors [27]. In a recent study, inhibition of VEGF receptor 2 after ischaemia worsened injury and also affected cell death patterns with a shift from apoptosis to a necrosis phenotype [28]. In many other studies in which VEGF was administrated following stroke, the growth factor was shown to enhance brain repair processes [29, 30]. For all these reasons VEGF and its signalling of vasculogenesis has attracted much interest in recent years, revealing that neurogenesis is not the only process that responds to trophic factor therapy among possible brain repair therapies. Indeed, some trophic factors such as insulin growth factor-1, which has been reported to promote recovery after stroke [31–33], exert their activity in different routes by enhancing endothelial function, regulating apoptosis and having anti-inflammatory properties instead of just affecting neurogenesis [34, 35].

Another process that is modulated by brain repair therapies is myelin formation. Again, we emphasize the importance of connections between elements of the different pathways involved in brain repair after ischaemia. Recent publications have suggested connections within signal transduction pathways between elements such as Lingo-1 and epidermal growth factor [36]. Given that Lingo1 antibodies can promote recovery from demyelinating disease in animal models [37], trophic factors that might modulate Nogo-A or Lingo1 activities may offer interesting possibilities for brain repair. Important inhibitors of axonal remodelling, such as Nogo-A, are augmented after cerebral ischaemia [38] and their inhibition through viral-mediated RNAi enhances axonal connectivity [39]; therefore, strategies that enhance myelin formation and axonal remodelling through trophic factors are a possible way forward in stroke research.

Other possible treatment approaches are blood-mobilizing drugs like erythropoietin (EPO) [40–42] or Granulocyte colony-stimulating factor (G-CSF), which have been shown to have positive results in animal models [43, 44]. Interestingly, higher levels of neovascularization

Table 1 Main results of therapeutic studies with trophic factors or drugs with trophic effects in cerebral infarct animal models and human clinical trials

	Animal models	Clinical trials
Trophic factors		
Basic fibroblast growth factor (bFGF)	Promotes neurogenesis [18] Enhances functional recovery and stimulates progenitor cell proliferation [19]	Phase III (286 patients). Prematurely stopped [53]
Brain-derived neurotrophic factor (BDNF)	Cellular and functional recovery [21] Protects and promotes nerve fibreregeneration [22] Promotes prostacyclin biosynthesis [23]	No studies
Vascular endothelial growth factor (VEGF)	Reduces neuronal cell death, increases angiogenesis and vascular permeability [116, 117] reduces infarct volume, improves behavioural recovery [30]	No studies
Erythropoietin (EPO)	Reduces infarct size and improves neurobehavioral deficits [41]	Safety: open label (13 patients); Efficacy: double-blind randomized proof of concept trial (40 patients): Improvement in neurological outcome, and smaller lesion size [55] Phase II/III (522 patients): negative results and safety concerns [56]
Granulocyte colony-stimulating factor (G-CSF)	Promotes new blood vessel formation, has anti-inflammatory, anti-excytotoxic, neuroprotective properties [43] and survival-enhancing capacity and effects on functional outcome [44]	Safety: Phase IIb (60 patients): [58] Safety and efficacy: AXIS-2 Trial finished. Results not yet published
EPO + G-CSF	Enhances angiogenesis and tissue plasticity, leading to greater functional recovery [45]	No studies
Drugs with trophic effects		
CDP-choline (citicoline)	Increases neuronal plasticity and contributes to sensorimotor function recovery [48] Promotes protective and repair mechanisms [46, 47, 49]	Efficacy and safety: Individual pooled data analysis [61] Efficacy: Phase III (ICTUS Trial; 2078 patients) finished [62]. Results not yet published
Porcine brain derived peptide (cerebrolysin)	Reduces infarct volume and improves recovery [50] with increased neurogenesis [51], efficacy in neurological recovery, reduction of neuronal death, increased cell proliferation and decreased inflammatory response [52]	Safety and efficacy: Phase II clinical trial (146 patients) [63] and Cochrane Syst Rev [64]: not enough evidence for efficacy. No safety concerns Safety and efficacy: Phase IV Clinical trial finished: CASTA (1070 patients) safety confirmed; possible efficacy in more severe strokes [66]

A non-systematic selection of the main results of therapeutic studies with trophic factors or drugs with trophic effects in animal models and clinical trials of cerebral ischaemia is provided. The reference number for each study is shown in brackets. Information from ongoing clinical trials can be consulted in the PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and clinical trials (http://clinicaltrials.gov/) databases.

and endogenous stem cell biological activity were observed when these factors were combined in a recent study [45]. In light of these results, an open question in trophic factor therapy is whether augmented efficacy could be obtained by applying these factors in specific combinations instead of using any one factor alone.

On the other hand, some other drugs with trophic effects, like CDP-choline, which have been attributed with a protective role [46, 47], have come under investigation. Although its mechanisms of action are unknown, it is thought that CDP-choline improves both the structural integrity and functionality of the neuronal membranes, and this may in turn assist membrane repair [48]. Experimental animal studies have demonstrated that CDP-choline not only promotes protective mechanisms (decreasing gliosis and cell death) but also seems to stimulate repair (increasing endogenous cellular proliferation, angiogenesis and synaptogenesis) [49]. Another drug that has been attributed with a protective role is Cerebrolysin, which has exhibited trophic properties when applied 24 and 48 hrs after stroke in animal models of ischaemia [50]. Indeed, while infarction volume does not seem to be substantially reduced with this treatment, the functional outcome is improved and proliferation, migration and

survival of neuroblasts, especially in the peri-infarct area, have been thought to contribute to the observed results [51, 52].

Clinical studies

Some of the trophic factors mentioned above, including bFGF, EPO and G-CSF, have been tested in controlled human clinical trials (Table 1). Unfortunately and in contrast with the experimental studies, the results have been mixed. As an example, a phase III clinical trial for intravenous bFGF administration for acute ischaemic stroke was stopped because an interim analysis of efficacy data predicted too small a chance of demonstrating a statistically significant benefit. In addition, unexpected peripheral side effects including leucocytosis and decreased blood pressure were reported in the treated group [53]. Nevertheless, new trials are still being considered as the pre-clinical data continue to justify further controlled clinical research with larger cohorts of patients [54].

In the case of EPO, the results are also inconclusive. While an initial small-size proof of concept trial performed in acute stroke patients reported an improvement in stroke outcome at 1 month and significantly smaller lesion size in the treated group than in controls without relevant side effects [55], a larger phase II/III study with EPO ended with negative results and safety concerns [56]. The possibility that some of these factors could be especially interesting for specific stroke subtypes is also not clear at the moment. Peripheral blood mobilization factors like G-CSF have been tested in humans and have been found to be safe [57, 58]. In addition, an exploratory analysis has suggested dose-dependent beneficial effects from G-CSF treatment in patients who had large baseline diffusion-weighted image lesions, supporting further investigation and repeated trials with larger cohorts of patients [59]. An AXIS-II trial investigating the safety, tolerability and effect of G-CSF in acute ischaemic stroke patients has recently completed recruitment but results have not yet been published [60].

Based on their pre-clinical results, other drugs with trophic effects, specifically CDP-choline and Cerebrolysin, have also been tested in clinical trials. A meta-analysis of pooled data collected from several small phase III trials, concluded that CDP-choline was reported to be safe and present a certain efficacy. In patients with moderate to severe ischaemic stroke, oral CDP-choline for 6 weeks increased the global odds of recovery at 3 months by 33% compared with the placebo [61] and this is being studied more thoroughly in a larger clinical phase III trial (ICTUS) [62], which has recently completed recruitment with results to be published soon [60]. In the case of Cerebrolysin, a randomized placebo-controlled trial (146 patients) showed a significant improvement of cognitive function of patients treated with Cerebrolysin, but without a significant effect on neurological or functional outcome [63]. A systematic Cochrane review reported not enough evidence to evaluate the effect of cerebrolysisn on survival and dependency in acute ischaemic stroke with no safety concerns [64], a large double-blind placebo-controlled randomized phase III clinical trial (1070 patients) conducted in Asiatic patients (CASTA), confirmed its safety and suggested a benefit for the group with more severe strokes [65, 66]. Thus, it could be interesting to continue the research development of this drug.

In summary, to date there is insufficient knowledge of efficacy of trophic factors in ischaemic stroke based on clinical trials, and the publication of the results of the AXIS-II trial should give more information. On the other hand, research on drugs with trophic effects has demonstrated the safety of CDP-choline and of Cerebrolysin, and suggests some efficacy in acute ischaemic stroke.

Stem cell therapies

As well as immune-modulation and substitution of damaged areas under certain conditions [27, 67], the available evidence supports the concept that stem cells assist recovery by modulating brain repair processes, including neurogenesis, gliogenesis, synaptogenesis and angiogenesis. While the molecular events underlying these processes are mostly unknown, it has been suggested that stem cells are capable of secreting trophic factors (VEGF, bFGF, BDNF) [68], in response to repair processes amplifying their levels in the brain. After culture in *ex-vivo* experiments, trophic interactions between MSCs and ischaemic brain have led to increased production of trophic factors including BDNF, VEGF or HGF (hepatocyte growth factor) [69] and it is known that stem cells express receptors that might allow these interactions [70, 71]. Furthermore, it has been discovered that stem cell transplantation is more effective when implanted cells are derived from stroke animals than when harvested from controls [72]. This recent report supports the hypothesis of trophic interactions between damaged brain and stem cells under ischaemic conditions that would prepare stem cells to exert their positive function. Interestingly, if these trophic interactions exist, another treatment strategy might be to combine stem cell therapy methods with trophic factor pre-treatment *in vitro* before their application. Under this perspective, stem cells genetically modified to overexpress specific trophic factors might enhance neuronal differentiation and survival [73]; in addition, gene modification of MSCs using viral vectors or RNA-based techniques may be a key to obtaining enhanced expression of specific desired factors (*i.e.* FGF-2) in comparison with 'wild type'-MSC transplantation [74].

Four major aspects will be reviewed below, concerning: (i) stem cell sources; (ii) the type of cell transplant, based on cell source; (iii) the time of administration or therapeutic window; and finally, (iv) the most suitable administration route for its clinical translation.

Looking at the wide range of stem cell sources, cerebral ischaemia can be treated using different types of cells from different origins (see Table 2). Enhanced function has been reported with different cell populations under different experimental conditions [75, 76]. Although a variety of conditions have been proposed for cell therapy, there is still no proven stem cell-based approach for stroke treatment and substantial symptomatic relief has not yet been demonstrated in patients [67, 76, 77].

Experimental animal models

In experimental animal models, neural stem cell (NSC) administration has been shown to enhance axonal sprouting and transport, dendritic activity and the expression of neurogenesis, gliogenesis and

Table 2 Brief summary: stem cell types

ESC (embryonic stem cells): Pluripotent self-renewing stem cells derived from the inner cell mass of embryos
IPS (inducible pluripotent stem cells): Adult somatic stem cells derived from normal adult tissues modified through genetic engineering; They resemble pluripotent stem cells and have self-renewing potential
NSC (neural stem cells): Self-Renewing cells capable of differentiating into the most relevant brain cell types (neurons, astrocytes, oligodendrocytes)
BMSCs (bone marrow stem cells)
HSCs (hematopoietic stem cells, CD34+). Heterogeneous populations of multipotent cells capable of differentiating into all blood cell types (both myeloid and lymphoid)
EPCs (endothelial progenitor stem cells, CD34+). Circulating blood cells capable of differentiating into endothelial cells (angiogenesis)
MSCs (Bone Marrow Mesenchymal Stem Cells, CD34−). Multipotent stem cells from circulating blood with recently discovered reparative potential in damaged tissues.
MSC (mesenchymal stem cells)
ASC (adipose-derived MSCs). Mesenchymal stem cells highly concentrated in adipose tissues
pMSC (placental MSCs). Mesenchymal stem cells from the placenta
UCBs (umbilical cord blood MSCs). Mesenchymal stem cells in umbilical cord blood

neurotrophic support-associated genes [78–81]. While infarct size is not significantly reduced, levels of cell death and Bax-positive cells are decreased after 7 days of treatment in these experimental animals while Bcl-2 expression in the penumbra is augmented and neurological function is improved [82]. This is also observed when using other non-neural stem cell sources such as bone marrow (BMSC), umbilical cord blood cells (UCBC) or mesenchymal stem cells (MSC). Indeed, bone marrow mononuclear cell (BMMC) transplantation can promote proliferation of the endogenous NSCs and this is observed concomitantly with increased proliferation of endothelial cells (angiogenesis) following ischaemic stroke [83]. Endogenous NSC can be found around the peri-infarct area adjacent to endothelial cells, so it has been suggested that at least some NSCs are originated from microvascular pericytes. The mechanisms involved in the endogenous neurogenesis and vasculogenesis after BMC administration are still unclear and therefore under investigation.

Meanwhile, bone marrow-derived MSC [84] also hold great promise for cell therapy. The beneficial effects of MSC administration in experimental animal stroke models is well-described and there are a variety of studies with similar good results in structural/functional recovery [73, 85, 86]. A recent review summarizes the role of therapeutic mobilization of transplanted bone marrow stem cells and its importance for brain plasticity and remodelling in stroke [87]. Adipose tissue like bone marrow, is another source of MSC in which interest is growing because it provides an abundant, ethically unproblematic and accessible source of cells with similar potential to that of other adult stem cells [88, 89]. The same can be said of placenta cells, which also have low immunogenic properties and are easily obtained [90, 91]. *In vivo*, bioactive molecules secreted by MSCs provide a regenerative microenvironment that enhances a self-regulated regenerative response. This regenerative microenvironment (trophic activity) mediates tissue repair and regeneration under ischaemia conditions [92].

As was previously mentioned, endothelial cell regeneration and neovascularization after tissue ischaemia are subjects of interest nowadays in the context of brain repair and it has been reported that repair can be enhanced by the administration of endothelial progenitor cells (EPCs), the positive effects of which have been observed in long-term neurobehavioural tests [93, 94].

Lastly, another ethically unproblematic source of cells with great potential are inducible pluripotent stem cells (iPS). First described by the Yamanaka group [95], this kind of approach, combined with transplantation onto biodegradable matrices could provide an interesting framework for stem cell-based therapies [27]. In previous reports, iPS treatment has been shown to improve motor function, reduce infarct size, attenuate inflammatory cytokines and mediate protection [96]. However, as a therapeutic option, iPS cells require further evaluation in light of their high tumourigenic potential under certain conditions, a major concern for clinical use [67, 97].

Independently of the above-mentioned cell sources, an important practical issue is the type of transplant: autologous (same individual), allogenic (same species) or xenogenic (another species). To prevent rejection, autologous administration can be considered the best option. A limitation of this approach in a clinical situation is that it would only allow treatment several weeks after the stroke, as this is the time needed for the cultivation and expansion of cells from the donor [98]. However, as the most appropriate time for administering stem cells is not clear and pre-clinical data also indicate that acute allogenic administration is both safe and effective [99], it might be possible to consider the creation of biobanks of allogenic stem cells (donors) to treat cerebral infarct patients earlier, within the acute phase time period.

To emphasize results from a clinical perspective, the route of administration for stem cells is still a major concern. There are several possible options which have been tested in experimental animals including intrastriatal, intraventricular, intravenous [100], intracarotid [101] or intranasal routes [102]. Some of them have an apparently similar effectiveness, but intravenous administration would be the least invasive delivery mode for use in future clinical applications [99]. However, as stroke is a localized CNS disease, new options and ideas in CNS-directed delivery are desirable. Also, new implantation sites such as the epi-cortical implant, a new minimally invasive method [103] or also the plexus-CSF route [104], would minimize or eliminate the distribution of graft cells to peripheral organs and obviate the need for a surgical (cell) implantation that is required by the

intracarotid route. Either way, studies in experimental animals should focus on imaging and cell tracking of the transplanted cells.

Taking into consideration that in patients acute stroke is usually considered a 'time is life' condition, the time of transplant is critical, and, for now, there is no clear concordance between animal models and humans. It has been described that the blood-brain barrier is open continuously for several weeks after ischaemia [105], indicating that the injured tissue may permit the entrance of exogenous cells during a long post-ischaemic window and this possibility has resulted in most studies having been focused on post-acute MSC administration [73]. However, with these long experimental conditions it is not possible to evaluate the protective effects, if any, of these cells and whether early administration might interact with reparative modulation in the brain. Furthermore, gliogenesis could also have a detrimental role as glial scarring in the late state of cerebral infarct may impede or compromise the delivery of new cells to the peri-infarct areas where they could exert their positive function.

Clinical studies

Globally and in contrast to results from experimental animal models (Table 3), clinical trials with stem cells have reported safety but mixed results in terms of efficacy [106]. As an example, cultured human (h) NSCs stereotactically implanted in patients with motor deficits, did not produce evidence of a significant benefit in terms of motor function although safety and feasibility was confirmed [107].

With regards to MSCs, some studies have reported that they can be safely transplanted into the brain of patients with excellent tolerance and without complications [108]. Also systemically, in patients with severe cerebral infarcts, intravenous infusion of autologous MSCs appears to be a feasible and safe therapy [109] that may improve functional recovery [98]. A long-term follow-up study has shown the safety of the treatment after 5 years [110], this kind of positive result is interesting and the study should be replicated with larger cohorts of patients.

Currently, there are open clinical trials using MSCs in ischaemic stroke. Phase I and phase II studies exist for BMMCs and results will be obtained soon. Other studies are evaluating the feasibility and tolerance of the intravenous injection of autologous MSCs in phase II and another 2 clinical trials in phase I/II are evaluating the intravenous injection of allogenic MSCs [60]. There are considerable difficulties in designing future efficacy trials, some of which are inherent to the field of regenerative treatment in stroke, and others specific to stem cells or their mode of delivery [111].

As has been discussed above, multiple subtypes of stem cell therapies have been developed in recent years for the treatment of cerebral ischaemia. However, large and well-designed trials are needed to identify the best options for their transfer to the clinical setting [112]. In 2007, the Stem Cell Therapy as an Emerging Paradigm for Stroke

Table 3 Main results of stem cells in animal models and cerebral infarct clinical trials

	Animal models	Clinical trials
Stem cells		
Neural stem cells (NSCs)/ neuronal cells	Promotes behavioural recovery and endogenous neurogenesis [79], reduces infarct volume [80] enhances axonal sprouting and the expression of genes involved in neurogenesis, gliogenesis, and neurotrophic support; modulates microglial response [118]. Anti-apoptotic activity [82]	Phase II (18 patients): No evidence of a significant benefit in motor function but safety and feasibility demonstrated in [107] Safety of a manufactured neural stem cell line (CTX0E03) is being tested (PISCES study, Phase I)
Mesenchymal stem cells (MSCs)	Enhances structural/functional recovery [85], reduces lesion volume, decreases inflammatory cell proliferation [86, 88]	Stereotactic implantation: Safety: Open study (5 patients): with excellent tolerance [108] Intravenous administration: Safety: Open label (12 patients): no safety concerns [109] Safety and efficacy: Phase I/II (30 patients) no adverse events and better outcomes in MSC-treated patients [98] Open label long-term follow-up (52 patients): safe and clinical improvement [110]
Bone marrow stem cells (BMSCs)	CD34: enhanced neovascularisation, neurogenesis, functional recovery [119] EPCs: protected the brain against ischaemic injury, promoted neurovascular repair and improved long-term neurobehavioural outcomes [93]	Safety: Ongoing Phase I and Phase II trials. CD34: autologous CD34+ subset BMSC infusion and intercerebral implantation

Main results of stem cell therapy in animal models and clinical trials of cerebral ischaemia. The reference number for each study is shown in brackets. Information from ongoing clinical trials can be seen in the PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and Clinical trials (http://clinicaltrials.gov/) databases.

(STEPS) meeting was organized to accelerate the field of cell therapy for stroke and to address outstanding questions [113]. In 2010, a second meeting, STEPS2, was held. Participants identified critical gaps in knowledge and research areas that require further studies, updated existing guidelines and drafted new recommendations to create a framework to guide future investigations into cell-based therapies for stroke [114]. In summary, larger trials with stringent and well-delimited inclusion criteria are necessary as the results from preclinical studies still support significant beneficial effects from cell therapy. Furthermore, a better understanding of cell fate following infusion in patients is desirable.

In our opinion, as suggested in a recent review by our group [115], and based on the lack of expression of MHC class II antigens, the use of allogenic mesenchymal stem cells [99] may broaden therapeutic interest in their use. This type of cell has been shown to be a good alternative for treating patients with cerebral infarction in the acute phase. Also the IV administration route is the least invasive and may offer the most suitable strategy for its clinical translation. Their administration during the acute phase could help to inhibit the first steps of the ischaemic cascade after stroke and enhance endogenous mechanisms of brain repair. Therefore, for the authors, the IV administration of mesenchymal stem cells in the acute phase amplifies the resources for good functional recovery and may be an effective therapy in the future.

Last comments

In cerebral ischaemia, protection and brain repair mechanisms are activated and orchestrated as a continuum once the disease process is initiated. Both trophic factors and stem cell therapy have been shown to modulate genetic and molecular programs underlying neuronal cell survival and axonal connectivity, angiogenesis, oligodendrogenesis and modulation of inflammation. Both therapeutic approaches have consistently shown exciting results in experimental animal models but their efficacy in patients has not yet been confirmed. Although not entirely understood, recent data have demonstrated paracrine interactions between stem cells and trophic factors, and this could suggest a multi-modal strategy for brain repair. Experimental research in the coming years will be focused on combining both approaches. At the same time, protocolizing the clinical context, dose, times and routes of administration will help design more effective clinical trials.

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Conflict of interest

E. Díez-Tejedor has collaborated as a clinical advisor pre-clinical and clinical trial researcher or as a speaker with the following companies: Astra-Zeneca, Bayer, Bristol-Myers Squibb, Boehringer Ingelheim, Cellerix, Ferrer Grupo, Pfizer, Sanofi, Sygnis Pharma AG and EVER Neuro Pharma.

B. Fuentes has collaborated as a clinical trial researcher or as a speaker with the following companies: Astra-Zeneca, Bristol-Myers Squibb, Boehringer Ingelheim, Ferrer Grupo, Pfizer, Sanofi and Sygnis Pharma AG.

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Artículo número 2:

Brain-Derived Neurotrophic Factor Administration Mediated Oligodendrocyte Differentiation and Myelin Formation in Subcortical Ischemic Stroke

(2015) *Stroke* 46: 221-228

Original Contribution

Brain-Derived Neurotrophic Factor Administration Mediated Oligodendrocyte Differentiation and Myelin Formation in Subcortical Ischemic Stroke

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Background and Purpose—Translational research is beginning to reveal the importance of trophic factors as a therapy for cellular brain repair. The purpose of this study was to analyze whether brain-derived neurotrophic factor (BDNF) administration could mediate oligodendrogenesis and remyelination after white matter injury in subcortical stroke.

Methods—Ischemia was induced in rats by injection of endothelin-1. At 24 hours, 0.4 μg/kg of BDNF or saline was intravenously administered to the treatment and control groups, respectively. Functional evaluation, MRI, and fiber tract integrity on tractography images were analyzed. Proliferation (KI-67) and white matter repair markers (A2B5, 2',3'-cyclic-nucleotide 3'-phosphodiesterase [CNPase], adenomatous polyposis coli [APC], platelet-derived growth factor receptor alpha [PDGFR-α], oligodendrocyte marker O4 [O4], oligodendrocyte transcription factor [Olig-2], and myelin basic protein [MBP]) were analyzed at 7 and 28 days.

Results—The BDNF-treated animals showed less functional deficit at 28 days after treatment than the controls ($P<0.05$). Although T2-MRI did not show differences in lesion size at 7 and 28 days between groups, diffusion tensor imaging tractography analysis revealed significantly better tract connectivity at 28 days in the BDNF group than in the controls ($P<0.05$). Increased proliferation of oligodendrocyte progenitors was observed in treated animals at 7 days ($P<0.05$). Finally, the levels of white matter repair markers (A2B5, CNPase, and O4 at 7 days; Olig-2 and MBP at 28 days) were higher in the BDNF group than in the controls ($P<0.05$).

Conclusions—BDNF administration exerted better functional outcome, oligodendrogenesis, remyelination, and fiber connectivity than controls in rats subjected to subcortical damage in ischemic stroke. **(*Stroke*. 2015;46:00-00.)**

Key Words: brain-derived neurotrophic factor ■ endothelin-1 ■ subcortical stroke

After decades of research focused on the search for a treatment for cortical infarcts in experimental models in which the gray matter is most affected, a few translational studies are beginning to highlight the importance of considering the white matter component after stroke.[1] Not only are ≤25% of ischemic strokes in humans subcortical or lacunar and confined to white matter regions[2] but also cortical infarcts produce white matter injury. The high frequency of this damage motivates the search for an effective therapy to enhance the mechanisms underlying the repair of damaged white matter (axons and myelin) after a stroke.[2]

Trophic factors are emerging as a viable repair therapy in stroke, and they can strongly promote a favorable environment for cellular repair after brain injury.[3,4] One of the prominent trophic molecules is brain-derived neurotrophic factor (BDNF), which is secreted in an activity-dependent manner and crucially promotes synaptic regulation and axonal plasticity associated with learning, memory, and sensorimotor recovery.[4,5] Furthermore, in vitro and BDNF knockout studies have demonstrated that this trophic factor has direct effects on oligodendroglia, promoting the proliferation and differentiation of oligodendrocyte precursor cells (OPC) and myelination.[6–8]

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All of the above indicate that BDNF could exert a possible effect on oligodendrogenesis and remyelination after a stroke. Thus, the present study explored the possible effect of BDNF administration on white matter remodeling via oligodendrogenesis and myelinogenesis and whether this effect might correlate with functional recovery in an animal model of subcortical stroke.

Materials and Methods

Study Design

This translational study followed all stroke therapy academic industry roundtable and RIGOR guidelines in terms of randomization, blinding and statistical power.[9] In addition, the experiments were designed to minimize animal suffering in compliance with our medical school's Ethical Committee for the Care and Use of Animals in Research (EU directives 86/609/CEE and 2003/65/CE). A total of 74 adult Sprague–Dawley rats (200–250 g) were randomly distributed into 3 groups: sham: surgery without endothelin-1 injection+intravenous saline administration (n=24); control: subcortical stroke+intravenous saline administration (n=24); and BDNF animals: subcortical stroke+intravenous BDNF treatment (n=24). In the BDNF group, recombinant human BDNF (Peprotech, United Kingdom) was diluted in saline to a final volume of 1 mL and administered through the tail vein at a final dose of 0.4 μg/kg at 24 hours after surgery. A saline solution of the same volume was delivered to the control and sham animals. The rats were then randomly divided into 3 subgroups that were euthanized at 4 hours (n=4 per each group), at 7 days (n=10 per each group) or at 28 days (n=10 per each group). Two rats were excluded from the study because one died after surgery and the other died during the magnetic resonance procedure.

Endothelin-1 Subcortical Stroke Model

In all the animals, physiological parameters and body temperature were continuously monitored during surgery (online-only Data Supplement). To provoke white matter injury, a subcortical stroke was induced to preanesthetized rats after a small craniotomy by the injection of a potent vasoconstrictor, endothelin-1, using a stereotactic apparatus using stereotactic references (+0.4 mm anteroposterior, +3.5 mm lateral, +6 mm dorsoventral from the bregma). One microliter of endothelin-1 (0.25 μg/μL) was delivered at a final speed of 0.2 μL/m. Immediately after surgery, analgesia was provided to all groups by an intraperitoneal injection of meloxicam at 2 mg/kg.

BDNF Quantification After Treatment

BDNF was quantified by the human BDNF ELISA kit (ABCAM) and Western blot in brain tissue and serum in both the control and the treated animals at 4 hours, 7 days, and 28 days (online-only Data Supplement). Immunofluorescence (using a primary antibody anti-BDNF [1:1000, Millipore] and a secondary antibody antirabbit Alexa Fluor 594 [1:750, Invitrogen]; n=4 per group).

Functional Evaluation Scales

Functional evaluation was scored in all animals by a blinded observer before surgery and at 1, 3, 7, and 28 days after treatment. Motor performance evaluation was measured by beam walking test,[10] the rotarod test,[11] and the Modified Rogers Scale[12] (online-only Data Supplement).

In Vivo Analysis by MRI and Diffusion Tensor Imaging Tractography

To analyze whether the BDNF effect could be examined with in vivo imaging techniques, we studied the ipsilateral hemisphere at the site of the endothelin-1–induced lesion using T2-MRI, diffusion on apparent diffusion coefficient (ADC) maps, and tract connectivity using diffusion tensor imaging (DTI) tractography (n=6 of each group) at 7 and 28 days after treatment (online-only Data Supplement).

Cell Proliferation Analysis

KI-67 staining was analyzed on days 7 and 28, using 10 sections selected from each animal (n=6 animals per group; online-only Data Supplement).

To evaluate differentiation markers in proliferating cells, we double-stained KI-67–positive cells with 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase), A2B5, adenomatous polyposis coli (APC), and platelet-derived growth factor receptor alpha (PDGFR-α) by immunofluorescence. All the images were acquired as a maximum confocal projection.

Immunohistochemistry, Immunofluorescence, and Western Blot

CryoMyelin staining kit (Hito Biotech) that allows sensitive localization and visualization of the myelin fibers was performed on frozen sections. The mean region of interest intensity in the CryoMyelin staining was quantified using a Nikon Eclipse-Ti inverted microscope and NIS-elements software. The lesion zone was studied in more detail using immunofluorescence and Western blot as previously described (n=4 animals of each group).[11] CNPase, A2B5, oligodendrocyte marker O4 (O4), Nogo-A, myelin basic protein (MBP), and oligodendrocyte transcription factor (Olig-2) markers were studied at 7 and 28 days (online-only Data Supplement).

Statistical Analysis

The data are presented as mean±SEM. The Kruskal–Wallis test followed by the Mann–Whitney *U* test was used to compare data. Values of $P<0.05$ were considered significant at a 95% confidence interval; the data were calculated using statistical software programs SPSS 16 and GraphPad.

Results

BDNF Levels Were Increased After Treatment

ELISA analysis of serum BDNF showed that the levels were augmented at 4 hours after its intravenous administration (4.33±0.32 and 5.61±0.62; $P<0.05$; Figure 1A). BDNF was analyzed in the BDNF-treated group and in the control animals throughout the brain. At 4 hours, significant higher levels of BDNF were found in the area of injury in BDNF animals (6.67±0.43) compared with the controls (0.79±0.24; $P<0.05$). Also Western blot analysis detected higher levels of BDNF in the ischemic lesion in the treated animals (0.97±0.08) compared with the control group (0.79±0.24) at 4 hours after treatment ($P<0.05$). At 7 and 28 days after administration, the BDNF levels were not significantly different in the treated animals (0.28±0.03 and 0.20±0.08) compared with the control group (0.27±0.063 and 0.204±0.049), respectively (Figure 1B and 1C). Immunofluorescence for BDNF showed both intracellular and extracellular staining (Figure 1C).

BDNF Improved Functional Recovery in the Subcortical White Matter Injury Model

No significant differences were found in the functional outcome of the treated and control animals at 1, 3, and 7 days after treatment. However, compared with the control rats, 28 days after treatment the BDNF-treated rats showed significantly better rotarod test performances (67.25±25.10 and 103.00±16.10, respectively; $P=0.049$), beam walking (3.5±0.70 and 1.4±0.54,

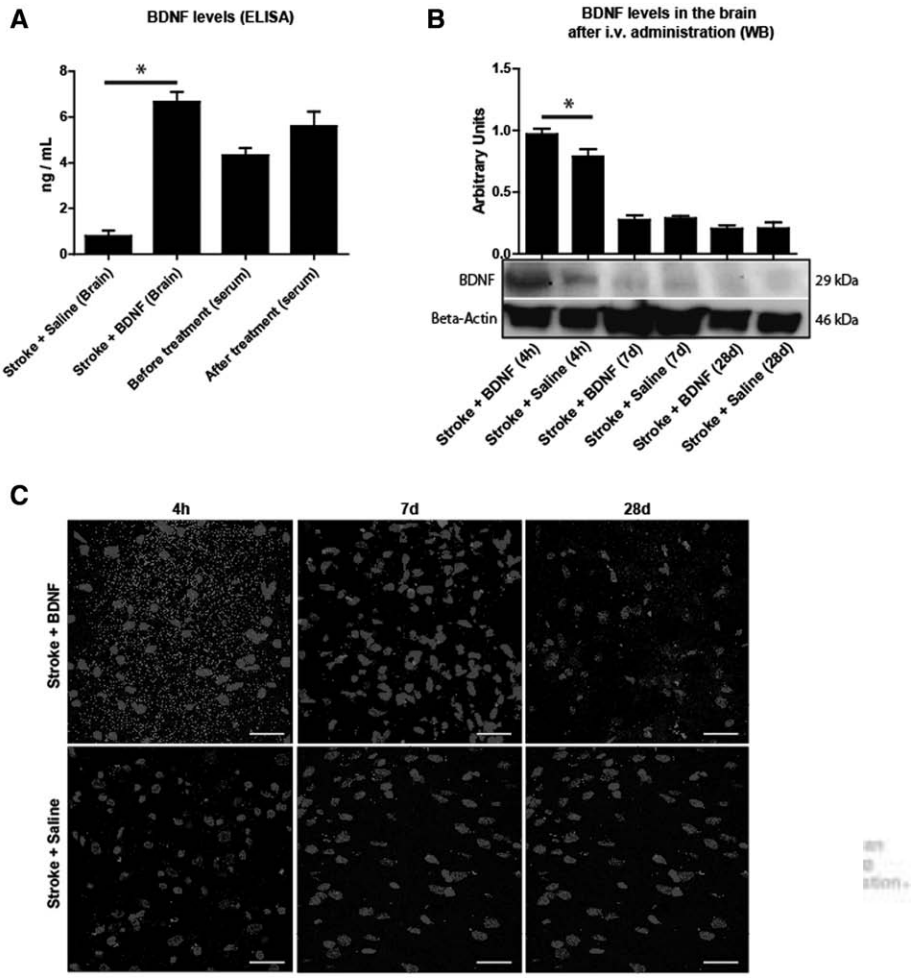

Figure 1. Brain-derived neurotrophic factor (BDNF) levels in the brain and peripheral serum are augmented at 4 hours after systemic BDNF administration returning to lower levels at 7 and 28 days. **A**, ELISA, (**B**) Western blot, and (**C**) immunofluorescence (IHF; data are mean±SEM; scale bars, 20 μm; *P<0.05, 4 animals, 4 sections each per group in IHF).

respectively; P=0.042), and Modified Rogers Test (2.5±0.57 and 1.2±0.44, respectively; P=0.018; Figure 2A).

BDNF Effects on White Matter Were Negligible by In Vivo MRI but Perceptible by Tractography and Myelin Staining

The lesion size viewed on MRI in the BDNF-treated animals was indistinguishable from the control group at 7 days (7.30±2.56 and 14.75±1.72, respectively) and 28 days (7.49±1.79 and 8.77±3.97, respectively) after treatment (P>0.05; Figure 2C).

DTI tractography data showed similar results in axial diffusivity (124.297±10.72 and 133.73±14.497; P>0.05) and diffusion in ADC maps (93.58±10.033 and 123.307±23.839; P>0.05) in both the control and the treated groups, respectively, at 7 days after stroke. However, compared with the control rats, 28 days after treatment the BDNF-treated rats showed significantly improved axial diffusivity (126.39±7.82 and 159.715±14.761, respectively; P<0.05) and diffusion in ADC maps (129.743±11.934 and 157.926±12.562, respectively; P<0.05; Figure 2C). This result suggests that there was a significant improvement in white matter thickness (width, breadth, and depth) and restoration of tract connectivity in the BDNF-treated animals compared with controls at 28 days.

These results are in agreement with the morphological study of myelin fibers by CryoMyelin staining. The mean region of interest intensity was calculated at the lesion site in the control and BDNF-treated animals, in which white intensity indicated absence of myelin and black intensity the presence of myelinated axons. The results showed higher intensity (absence of axons) in controls (198.61±26.30) compared with BDNF-treated animals (172.18±17.40; Figure 2B).

BDNF Administration Enhances OPC Proliferation After White Matter Injury

Numerous KI-67–positive cells were observed in the ischemic lesion in the control animals and in the BDNF-treated group. The number of KI-67–positive cells (170±17.32) was significantly higher in the ischemic lesion compared with control group (34.25±18.28; P=0.032) 7 days after BDNF treatment (Figure 3A).

Double staining of KI-67–positive cells with the OPC markers was observed to be higher in the subventricular zone in treated animals than in controls for A2B5 (9.12±1.87% and 5.45±2.10%), CNPase (31.00±7.32% and 22.12±5.34%), APC (26.00±5.12% and 17.32±4.21%) and PDGFR-α (36.00±6.23% and 28.00±7.34%), suggesting that OPC proliferation is enhanced after BDNF injection (Figure 3B).

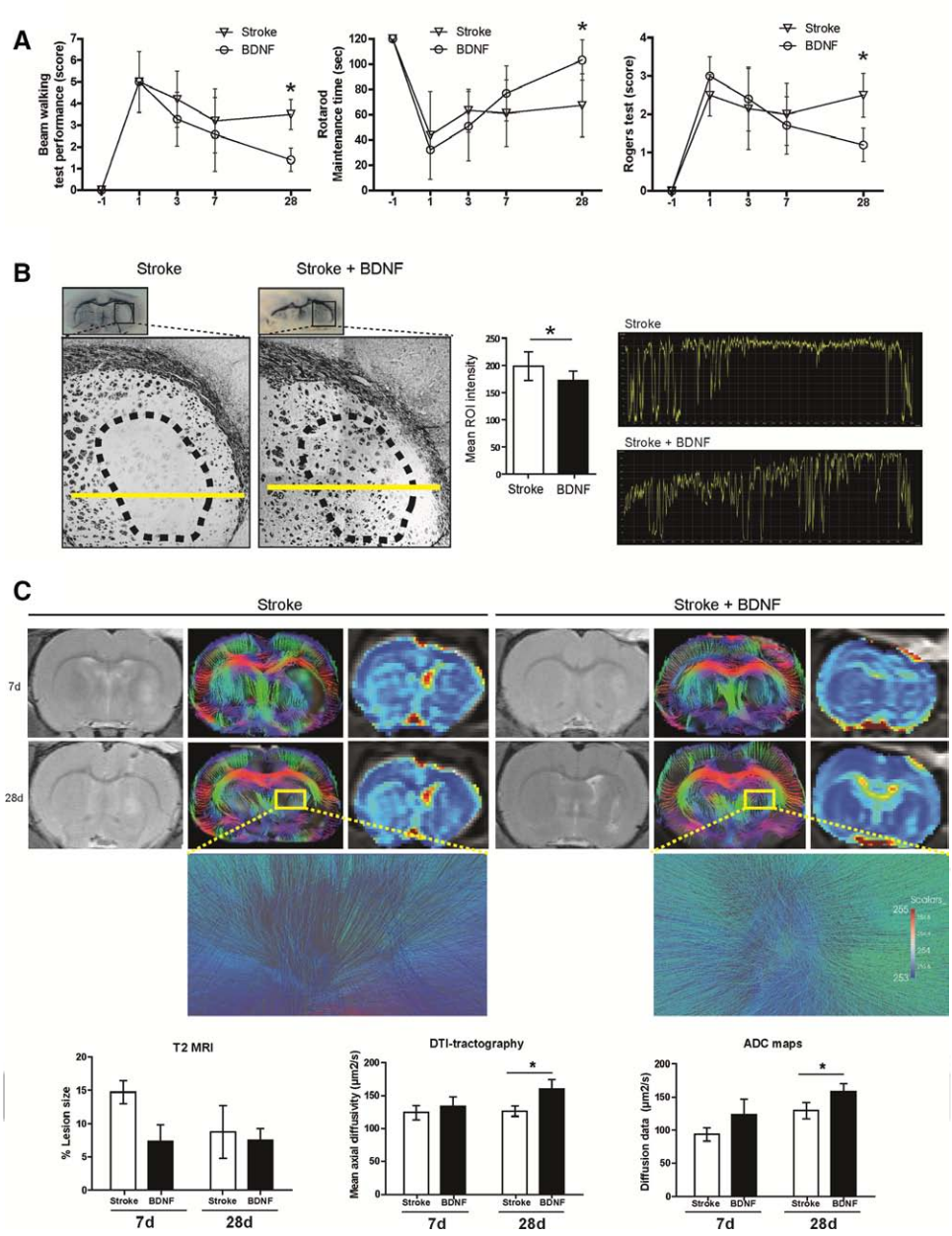

Figure 2. Improved functional recovery and connectivity in brain-derived neurotrophic factor (BDNF)–treated animals. **A**, Beam walking test (**left**), rotarod test (**middle**), and Modified Rogers Test (**right**). **B**, Morphological study by CryoMyelin staining identified the zone of the lesion as an area of white matter injury located in the subcortical zone, showing restored myelinated axons in the BDNF-treated animals. Quantification of mean region of interest intensity of the CryoMyelin staining Stroked line indicates region of interest; Yellow line indicates a representative longitudinal profile of pixel intensity. **C**, Qualitative and quantitative analysis of T2-weighted MRI (**left**), axial diffusivity in diffusion tensor imaging (DTI) tractography (**middle**), and diffusion in apparent diffusion coefficient (ADC) images (**right**) at 28 days showed a significant progressive reduction in white matter injury, showing augmented connectivity of the fiber tracts in the BDNF-treated animals compared with the control group. Zoomed lesion site 3-dimensional views of DTI tractography images are shown (data are mean±SEM; scale bars, 20 µm; **P*<0.05, 6 animals, 10 sections each per group).

BDNF Injection Increases OPC Markers 7 Days After Axonal Disruption

The levels of OPC markers were analyzed by Western blot in the lesion area at 7 and 28 days after treatment (Figure 4B). There was a significant increase in CNPase marker levels in the BDNF-treated animals (217.00±14.10) compared with the controls at 7 days (183.20±16.32; *P*<0.05). A2B5 was also higher in the BDNF-treated animals (141.20±41.20) than in the controls (75.39±13.20) at 7 days after treatment (*P*<0.05). We also found higher levels O4 marker at 7 days, in the BDNF-treated group (190.88±20.19) than in the control animals (82.10±18.01; *P*<0.05). At 28 days, the levels of OPC markers were lower in both control and BDNF animals. Also, when comparing treated and control groups at 28 days, no significant differences were found (A2B5 [91.12±41.10 versus 112.10±13.81], CNPase [89.23±21.10 versus 110.10±21.30], and O4 [75.20±19.10 versus 69.60±16.13], respectively). Immunofluorescence analysis confirmed these results (Figure 4A).

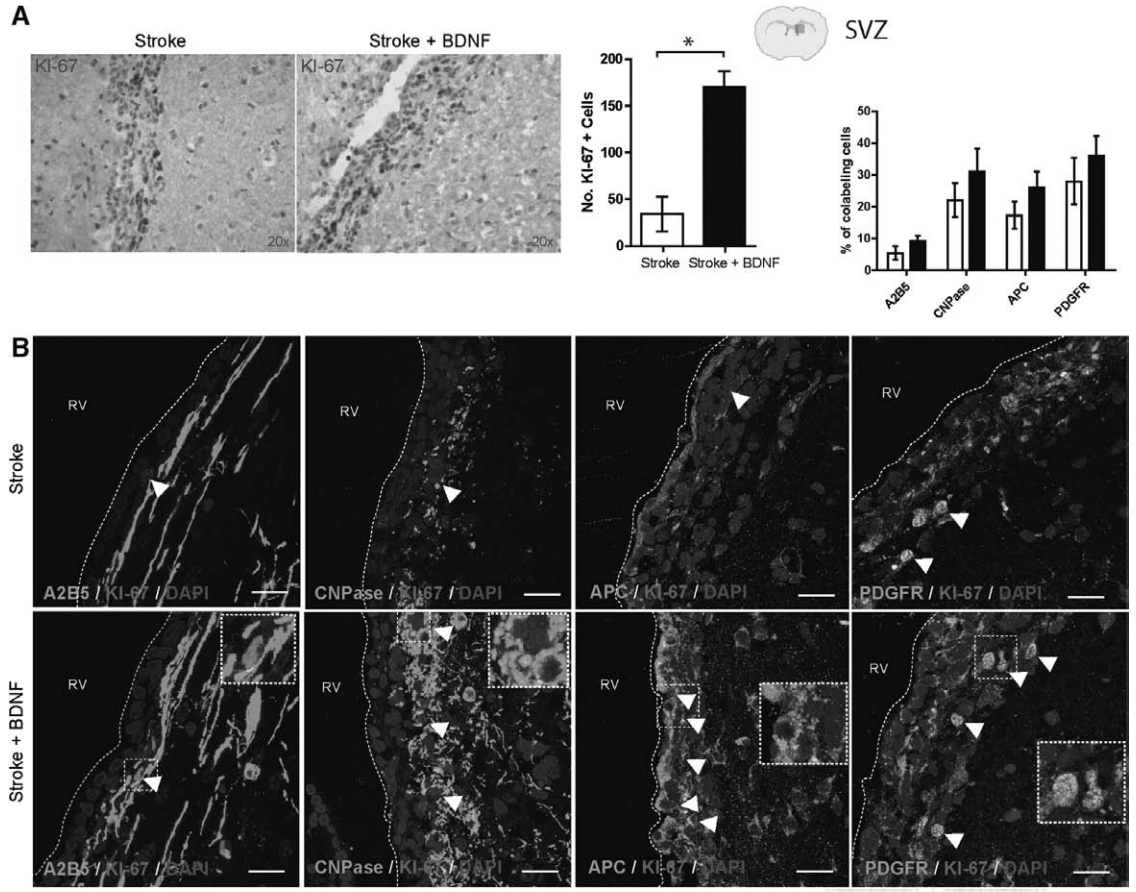

Figure 3. Augmented cell proliferation after brain-derived neurotrophic factor (BDNF) treatment at 7 days. **A**, Qualitative and quantitative analysis of cell proliferation by KI-67 staining. **B**, KI-67 colabeling with 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase), A2B5, adenomatous polyposis coli (APC), and platelet-derived growth factor receptor alpha (PDGFR-α) markers at 7 days after treatment (data are mean±SEM; scale bars, 20 μm, **P*<0.05, 6 animals, 10 sections each per group); 4',6-diamidino-2-phenylindole (DAPI) is used for nuclear staining. RV indicates right ventricle; and SVZ, subventricular zone.

BDNF Administration Enhances Oligodendrocyte Maturation and Axonal Growth-Associated Markers 28 Days After Injury

At 7 and 28 days, we analyzed markers related to later developmental steps in white matter differentiation (MBP, Olig-2) and in maturation of myelin fibers (Nogo-A; Figure 5A and 5B).

Western blot analysis showed significantly higher levels of the oligodendrocyte marker Olig-2 in the BDNF-treated animals than in the control group at 28 days (242.85±15.50 and 94.21±10.20, respectively; $P<0.05$) and a significant increase in MBP in the treated animals compared with the controls (192.18±29.99 and 106.55±26.74, respectively; $P<0.0.05$). We also found a significant decrease in Nogo-A levels in the BDNF-treated animals compared with the controls at 7 days (160.11±17.23 and 213.21±12.41, respectively) and at 28 days (163.01±18.88 and 200.10±9.87, respectively; $P=0.014$). At 7 days, the levels of Olig-2 (150.25±19.43 and 70.91±21.50) and MBP (51.21±23.10 and 25.01±10.80) were too low compared with 28 days in both the treated group and the control group because all these markers are related to later developmental steps in white matter differentiation.

Discussion

In the current search for new therapeutic strategies to improve functional and cognitive deficits after stroke, it is worth remembering that myelination failure prevention is necessary for brain repair processes. Thus, the present study used an intravenous infusion (0.4 μg/kg) of BDNF as a therapeutic strategy to prevent myelination failure. The group treated with BDNF injection showed improved functional recovery and a significant increase in the number of proliferating cells, including OPC, after white matter injury. After using BDNF, large numbers of cells expressed OPC markers, such as CNPase, A2B5, and O4 at 7 days. At 28 days after treatment, the cells began to acquire specific markers of oligodendrocyte differentiation, such as Olig-2 and MBP, suggesting that repair of white matter fiber tracts was induced by the BDNF injection. The results support a role for BDNF in improving the repair of white matter and in OPC proliferation and differentiation in this experimental subcortical stroke model.

This treatment was chosen because systemic administration is already known to allow BDNF to cross the blood brain barrier.[4,13] In this sense, the BDNF levels in our study were augmented in brain tissue and peripheral serum at 4 hours after injection. This increase of BDNF levels observed in the brain might enhance recovery mechanisms after stroke.

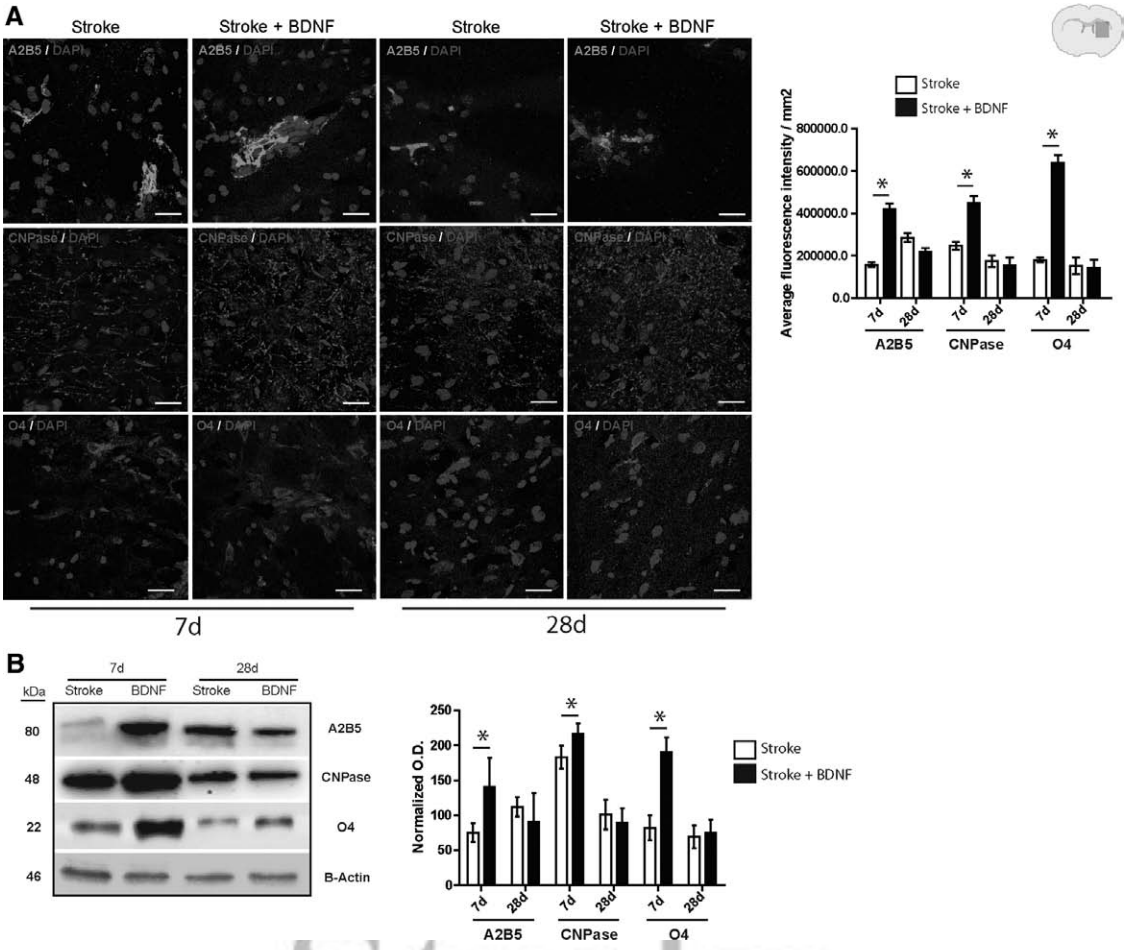

Figure 4. Increased OPC-related markers (A2B5, 2',3'-cyclic-nucleotide 3'-phosphodiesterase [CNPase], and oligodendrocyte marker O4 [O4]) at 7 days after brain-derived neurotrophic factor (BDNF) treatment. At 28 days, all these levels were decreased when compared with 7 days measured by immunofluorescence (**A**) and Western blot (**B**; data are mean±SEM; scale bars, 20 μm; **P*<0.05, 4 animals, 4 sections each per group); 4',6-diamidino-2-phenylindole (DAPI) is used for nuclear staining.

BDNF as therapy to induce brain protection and brain repair is becoming more common in translational research. After a cortical stroke, BDNF has been shown to control neuronal circuits, increase the number of newborn neurons in several brain areas,[3,4] reduce astrogliosis, enhance axonal growth in the ischemic border zone,[5] and stimulate the plasticity of dendritic branching and synaptic transmission.[14] However, the effects of BDNF administration on nerve fiber repair, myelin formation, and remodeling after white matter injury are still unknown.

In a translational study, it is important to analyze whether BDNF injections act on the motor dysfunctions that are characteristic to subcortical stroke. Previous authors found that the beam walking test,[10] the rotarod test,[11] and the Modified Rogers Test[12] to be effective in assessing the motor deficit associated primarily with subcortical stroke. In this study, BDNF treatment induced a significant improvement in functional recovery that was particularly notable at 28 days after treatment when compared with the controls. These results clearly suggest a true recovery-enhancing effect for BDNF. Although there are no previous studies that have administered BDNF in a subcortical stroke model, our results are consistent with previous data showing a better functional outcome in animals treated intravenously with BDNF after cortical ischemia.[4]

Focal injection of the vasoconstrictor endothelin-1 into the subcortical white matter produces a visible infarct on MRI, as previously shown in subcortical stroke studies.[15,16] To analyze whether the BDNF effect could be visualized with in vivo imaging techniques, we performed T2-MRI, ADC maps, and DTI tractography. No significant differences in lesion size were observed in the BDNF-treated animals compared with the control group on the T2-MRI images; however, analyzing fiber tract integrity by DTI tractography showed that tract thickness was recovered at 28 days after BDNF administration. This result suggests that functional recovery at 28 days could be related to the process by which restructured axons, which had previously been compromised and demyelinated, recover not only their proper structure but also tract connectivity. The lack of a relationship between the T2-MRI and DTI tractography images, however, remains unclear. This is why we intensified the histological analysis of the injured tissue in this study.

After a stroke, myelin injury activates the OPC distributed throughout the white matter, which proliferate and migrate to the site of damage where they subsequently mature into myelinating oligodendrocytes that ensheath axons to form the myelin membrane.[1,17] Our study using colocalization of the KI-67 antigen and the CNPase, A2B5, APC, and PDGFR-α markers showed

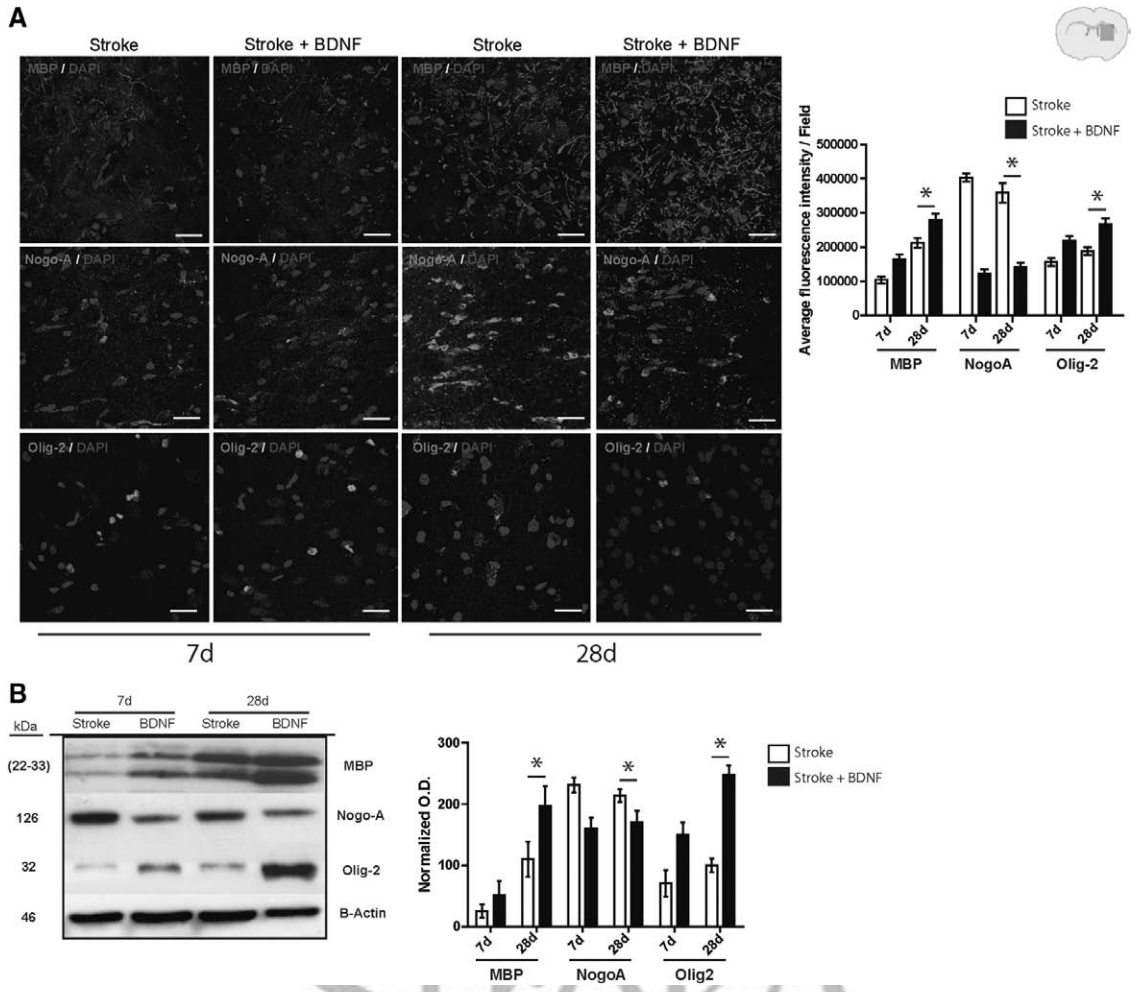

Figure 5. Increased white matter maturation and axonal growth-associated markers at 28 days (myelin basic protein [MBP], oligodendrocyte transcription factor [Olig-2]) and decreased levels of Nogo-A after BDNF treatment. **A**, Immunofluorescence and (**B**) Western blot (data are mean±SEM; scale bars, 20 μm; *P<0.05, 4 animals, 4 sections each per group); 4',6-diamidino-2-phenylindole (DAPI) is used for nuclear staining.

BDNF to be a potent factor for enhancing the OPC proliferation response after white matter injury. Our findings agree with other nonstroke studies in vitro and in BDNF knockout mice, showing that BDNF exerts direct effects on oligodendroglia, promoting OPC proliferation and differentiation, as well as myelination.[6–8] To elucidate whether BDNF administration also acts on oligodendrocytes and shattered white matter fibers and increases some repair mechanisms, including nerve fiber remodeling, axonal sprouting, oligodendrogenesis and myelinogenesis, we measured white matter repair-associated markers in both the treated and the control animals. Various white matter repair markers were studied at 2 different time points, 7 and 28 days. Related to the first steps of the genesis and migration of white matter progenitor cells, markers such as A2B5 (a characteristic OPC marker), O4, and CNPase (markers related to immature oligodendrocytes) were studied in the lesion area. Markers related to white matter differentiation and myelin fiber maturation such as MBP (myelin marker), Olig-2 (mature oligodendrocytes), and the myelin inhibitor Nogo-A were also analyzed.

The best-characterized oligodendroglial progenitor marker is A2B5.[18,19] This molecule is a cell surface ganglioside expressed on developing oligodendroglial progenitors. In our study, the levels of the A2B5 marker were higher in the BDNF-treated animals at 7 days than in the control group. O4 is another marker expressed in pro-oligodendrocytes during oligodendrocyte differentiation.[20] Our study found an increase in the amount of O4 protein in the animals subjected to BDNF administration compared with their controls. This observation agrees with a study describing the levels of CNPase.[19] The presence of CNPase seems to be one of the earliest events in oligodendrocyte differentiation, and BDNF-treated animals showed higher levels of this marker than the control group. The levels of A2B5, CNPase, and O4 were too low at 28 days because all these markers are related to early events in oligodendrocyte differentiation. These results are in line with previous nonstroke in vitro studies in which the effects of BDNF are examined in oligodendrocyte progenitors, finding enhanced A2B5 and O4 expression after neurotrophin administration.[21]

About the oligodendrocyte maturation-associated proteins, the levels of these markers were found to be too low at 7 days. However, Olig-2 levels were elevated in the treated animals compared with the control animals at 28 days, an observation that could be explained by BDNF having enhanced oligodendroglial cell differentiation. However, both the presence of mature

oligodendrocytes and coating the myelin sheath are important for white matter repair. There was a significant increase of MBP reactivity in the white matter tracts at 28 days in the treated animals when compared with the control group. Although there are no previous studies that relate BDNF to remyelination after stroke, these increases in the levels of MBP and Olig-2 concur well with previous nonstroke studies, indicating that BDNF influences differentiating oligodendrocytes by increasing both the number of MBP cells and the expression of the MBP protein.[22] What is unknown is whether the increased myelination was because more axons were being myelinated or whether there was an overall increase in myelin thickness.

Among the myelin-associated proteins, Nogo-A has been shown to be particularly powerful in preventing axonal growth and plasticity.[23] Nogo-A not only inhibits axonal growth but also prevents neurotrophins such as BDNF from binding to this receptor inhibiting axonal growth.[24] Some studies have demonstrated that inhibition mediated by Nogo-A is blocked if neurons are exposed to BDNF before encountering the inhibitor.[25] The present study concurs with these observations because at 28 days after treatment, there was a significant decrease in the levels of Nogo-A in BDNF animals. These results indicate that BDNF injection could enhance axonal growth and plasticity by decreasing Nogo-A levels. The observations found at 28 days suggest that functional recovery might be related to the axonal sprouting subsequent to BDNF administration and could be indicative of the process by which growing and as-yet demyelinated axons are recovered with new myelin sheaths. Furthermore, this interpretation of these observations agrees with the tractography images that suggest that tract connectivity was being restored in the infarcted area at the same time point.

Conclusions

Our study helped us to identify a clear role for BDNF in improving functional outcome by mediating axonal growth, OPC proliferation, oligodendrocyte differentiation, remyelination, and fiber tract connectivity restoration in an experimental animal model of white matter injury.

Acknowledgments

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

ET-1 subcortical stroke model

In all the animals, continuous monitoring of physiological parameters (glycemia, blood gases and blood pressure) was performed during surgery and ischemia (Monitor Omicron ALTEA RGB medical devices, Madrid, Spain). Body temperature was also monitored and maintained at 36.5 ± 0.5°C. All the surgical experiments were performed on adult rats that were secured on a stereotactic apparatus under isoflurane inhalatory anesthesia.

BDNF quantification after treatment

The BDNF levels were quantified by a human BDNF ELISA kit (ABCAM), Western blot and immunohistochemistry in the brain tissue and serum 4h after treatment in both the control animals and the treated animals. The BDNF ELISA determination was performed according to the manufacturer's specifications. The standard curves and the samples were run in duplicate. The serum was diluted 1:20. For BDNF biodistribution analysis in the brain, 40µg of total protein from all the different isolated brain areas were added to the different ELISA sample wells. The absorbance was measured at 450nm with an Epoch microplate spectrophotometer (Bioteck). To analyze BDNF brain levels at different times, Western blot and immunofluorescence were performed 4h, 7 and 28d after injection in the lesion zone, using a primary antibody anti-BDNF (1:1000, Millipore) and a secondary antibody anti-rabbit Alexa Fluor 594 (1:750, Invitrogen). The slices were examined using a LEICA TCS SPE spectral confocal microscope (Leica Microsystems, Heidelberg, Germany) and the confocal images were analyzed using LEICA software LAS AF, version 2.0.1 Build 2043. All the images were acquired as a maximum confocal projection. For the Western blot, the units were normalized based on beta-actin levels (1:400, Sigma-Aldrich).

Functional evaluation scales

Functional evaluation was scored by a blinded observer on all the animals before surgery and at 1, 3, 7 and 28d after treatment by 3 different tests. First, the beam walking test measured the ability of rats to cross a wooden beam (2.5×2.5×80 cm). Scores were assigned as follows: score 0, traversed the beam with no foot slip; score 1, traversed with grasping of the lateral side of the beam; score 2, showed difficulty crawling across the beam but was able to traverse; score 3, required >10 seconds to traverse the beam because of difficulty in walking; score 4, unable to traverse the beam; score 5, unable to move the body or any limb on the beam; and score 6, unable to stay on the beam for >10 seconds. Second, the rotarod test measured the latency to fall from a rotating cylinder. Beginning three days before surgery, rats were trained on the accelerating (4 to 40 rpm) cylinder consisting of three sessions per day. The time each animal remained on the rotarod was measured twice per animal with a 15-minutes interval between each trial. The mean of the two trials was calculated. Third, a variant of the Modified Rogers Scale consisted of a 7-point behavioral rating scale: score 0, no functional deficit; score 1, failure to extend left forepaw fully; score 2, decreased grip of the left forelimb while tail gently pulled; score 3, spontaneous movement in all directions, contralateral circling only if pulled by the tail; score 4, circling or walking to the left; score 5, walked only when stimulated; score 6, unresponsive to stimulation with a depressed level of consciousness; and score 7, dead.

T2-MRI and DTI tractography

The BDNF effects were studied with *in vivo* imaging techniques, using T2-MRI and DTI tractography. First, the lesion size was analyzed at 7 and 28d after treatment using magnetic resonance imaging (MRI) (Bruker Pharmascan, Ettlingen, Germany, 7 Tesla horizontal bore magnets) and T2 maps (RARE 8 T2, 180° flip angle, 3 averages) as previously described.[1] The lesion area was then expressed as a percentage of the contralateral hemisphere, after correcting for brain edema. Second, for diffusion analysis, we used ADC maps. Third, for tractography, diffusion tensor data (DTI) were acquired at 7 and 28d after treatment with a spin echo single shot echo planar imaging (EPI) pulse sequence using the following parameters: TR/TE: 5000/35ms; a signal average of 10, a 30 noncolinear diffusion gradient scheme with diffusion weighting of b=1000 s/mm2 and b=0 s/mm2, and field of view 3.5x3.5 cm. There were a total of 496 slices, and the data was acquired using 30 directions. The data were acquired with a single shot EPI sequence on a 96x96 matrix, and zero-filled k-space to construct a 128x128 image matrix. The images were obtained with medInria, a multi-platform medical image processing and visualization software. Analysis of ADC and DTI-tractography data was performed in the lesion zone using n=6 animals per group. Zoomed lesion site 3D views of DTI tractography images are represented using ParaView 4.1.0 software.

Cell proliferation analysis

For the KI-67 staining, tissue slices were placed in a boiled citrate buffer (pH 6) and a non-specific binding was blocked for 1h with 3% normal goat serum (Vector Laboratories). Ki-67 was used as a primary antibody (1:100, Millipore), and the Vectastain Universal Quick Kit PK8800 was used as a secondary antibody (1:100, Vector Laboratories). The images were acquired using a 40× objective lens and the total number of positive cells were counted in a minimum of 10 different microscope fields using image analysis software.

To study the differentiation of the proliferating cells, we double stained KI-67-positive cells (1:100, Millipore) with 2',3'-Cyclic-nucleotide3'-phosphodiesterase (CNPase) (1:500, Sigma-Aldrich), A2B5 (1:500, Millipore), APC (CC1) (1:20, Abcam) and PDGFR-alpha (1:100, Abcam). The corresponding secondary antibodies were goat anti-mouse Alexa Fluor 488 and Alexa Fluor 594 (1:750, Invitrogen). The co-stained cells were observed using the 40× objective lens with LEICA software LAS AF. All the images were acquired as a maximum confocal projection.

Immunohistochemistry, immunofluorescence and Western blot

The staining of the myelin and myelinated axons was performed on frozen sections using a CryoMyelin kit (Hito Biotech, USA), which allows sensitive localization and visualization of the myelin fibers. The lesion zone was studied in more detail using immunofluorescence and Western blot, as previously described (n=4 animals of each group).[1] The different primary antibodies used for immunofluorescence and Western blot analysis were CNPase (1:500, Sigma-Aldrich), A2B5 (1:500, Millipore), Oligodendrocyte marker-4 (O4) (1:25, Millipore), Nogo-A (1:100, ABCAM), myelin basic protein (MBP) (1:100, ABCAM) and the marker oligodendrocyte marker-2 (Olig-2) (1:500, Millipore). The secondary antibodies for immunofluorescence were goat anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 (1:750, Invitrogen). The slices were examined using a LEICA TCS SPE spectral confocal microscope (Leica

Microsystems, Heidelberg, Germany) and the confocal images were analyzed using LEICA software LAS AF, version 2.0.1 Build 2043. All the images were acquired as a maximum confocal projection. To quantify the levels of brain markers by immunofluorescence, the experiments, images and quantification of the control and the BDNF samples were performed the same day, with the same microscope configurations and by blinded observers to eliminate bias due to background normalization. For the Western blot, the units were normalized based on beta-actin levels (1:400, Sigma-Aldrich).

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Brain-Derived Neurotrophic Factor Administration Mediated Oligodendrocyte Differentiation and Myelin Formation in Subcortical Ischemic Stroke

Jaime Ramos-Cejudo, María Gutiérrez-Fernández, Laura Otero-Ortega, Berta Rodríguez-Frutos, Blanca Fuentes, Maria Teresa Vallejo-Cremades, Teresa Navarro Hernanz, Sebastián Cerdán and Exuperio Díez-Tejedor

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Material Suplementario

Con el objetivo de mejorar la exposición del trabajo y, puesto que en la publicación original las figuras aparecen en blanco y negro, a continuación se facilitan las mismas en color y alta resolución para así clarificar su comprensión.

Figura 1

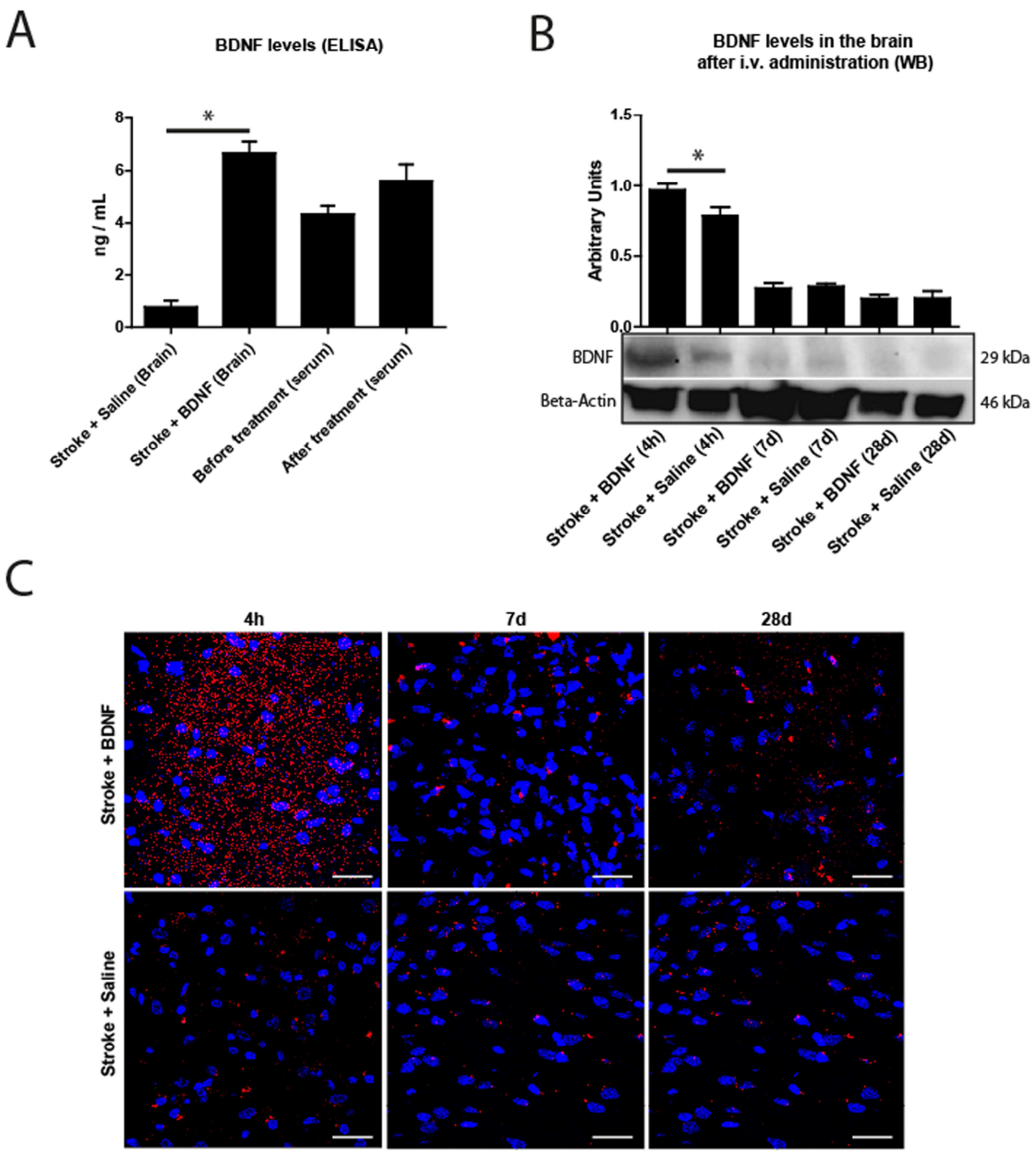

A
BDNF levels (ELISA)
ng / mL
8
6
4
2
0
*
Stroke + Saline (Brain)
Stroke + BDNF (Brain)
Before treatment (serum)
After treatment (serum)
B
BDNF levels in the brain
after i.v. administration (WB)
Arbitrary Units
1.5
1.0
0.5
0.0
*
BDNF
Beta-Actin
29 kDa
46 kDa
Stroke + BDNF (4h)
Stroke + Saline (4h)
Stroke + BDNF (7d)
Stroke + Saline (7d)
Stroke + BDNF (28d)
Stroke + Saline (28d)
C
4h
7d
28d
Stroke + BDNF
Stroke + Saline

Figura 2

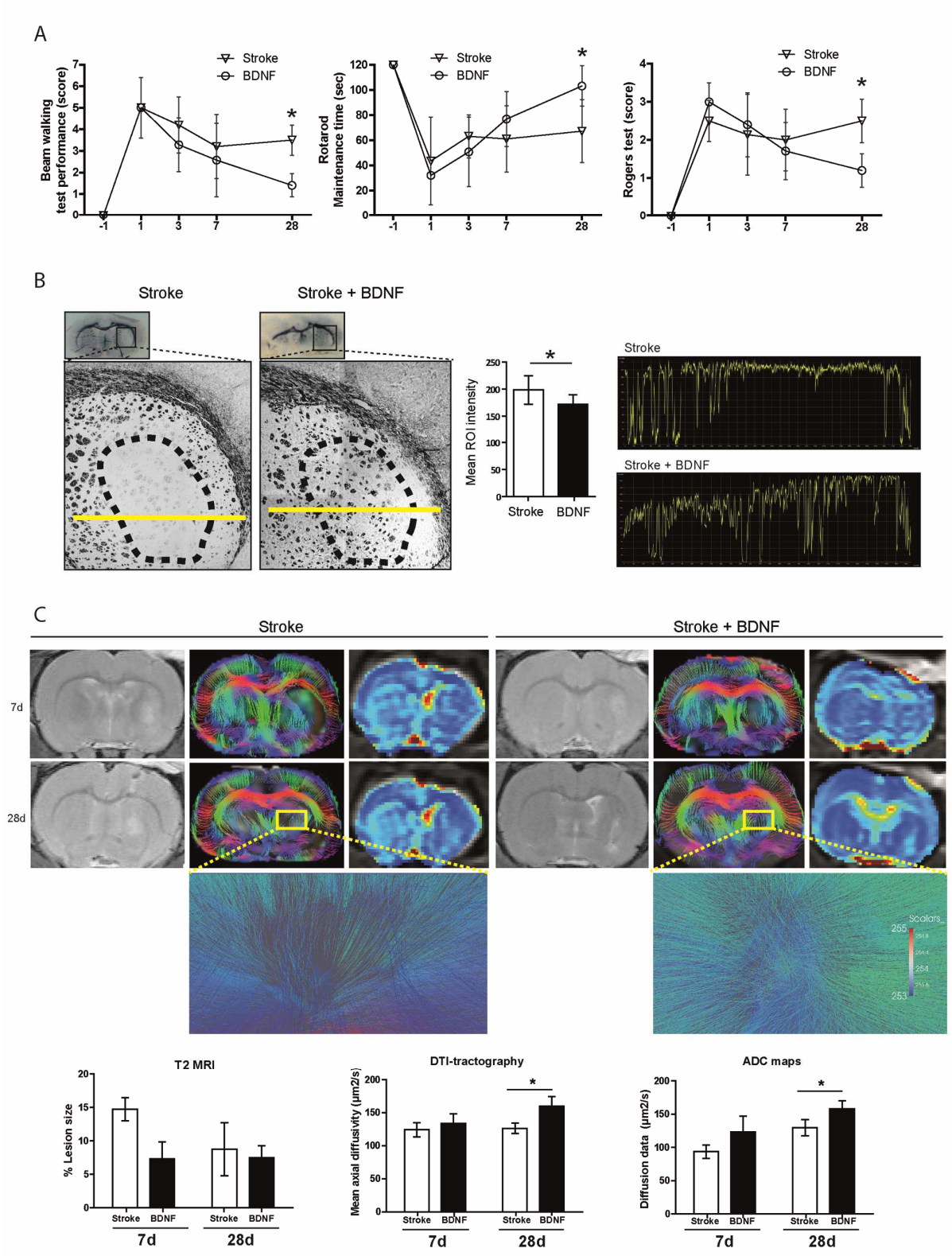

A
Stroke
BDNF
Beam walking test performance (score)
Rotarod Maintenance time (sec)
Rogers test (score)
-1
1
3
7
28
*
B
Stroke
Stroke + BDNF
Mean ROI intensity
C
7d
28d
Scalars
255
254
253
T2 MRI
% Lesion size
DTI-tractography
Mean axial diffusivity (µm2/s)
ADC maps
Diffusion data (µm2/s)
7d
28d

Figura 3

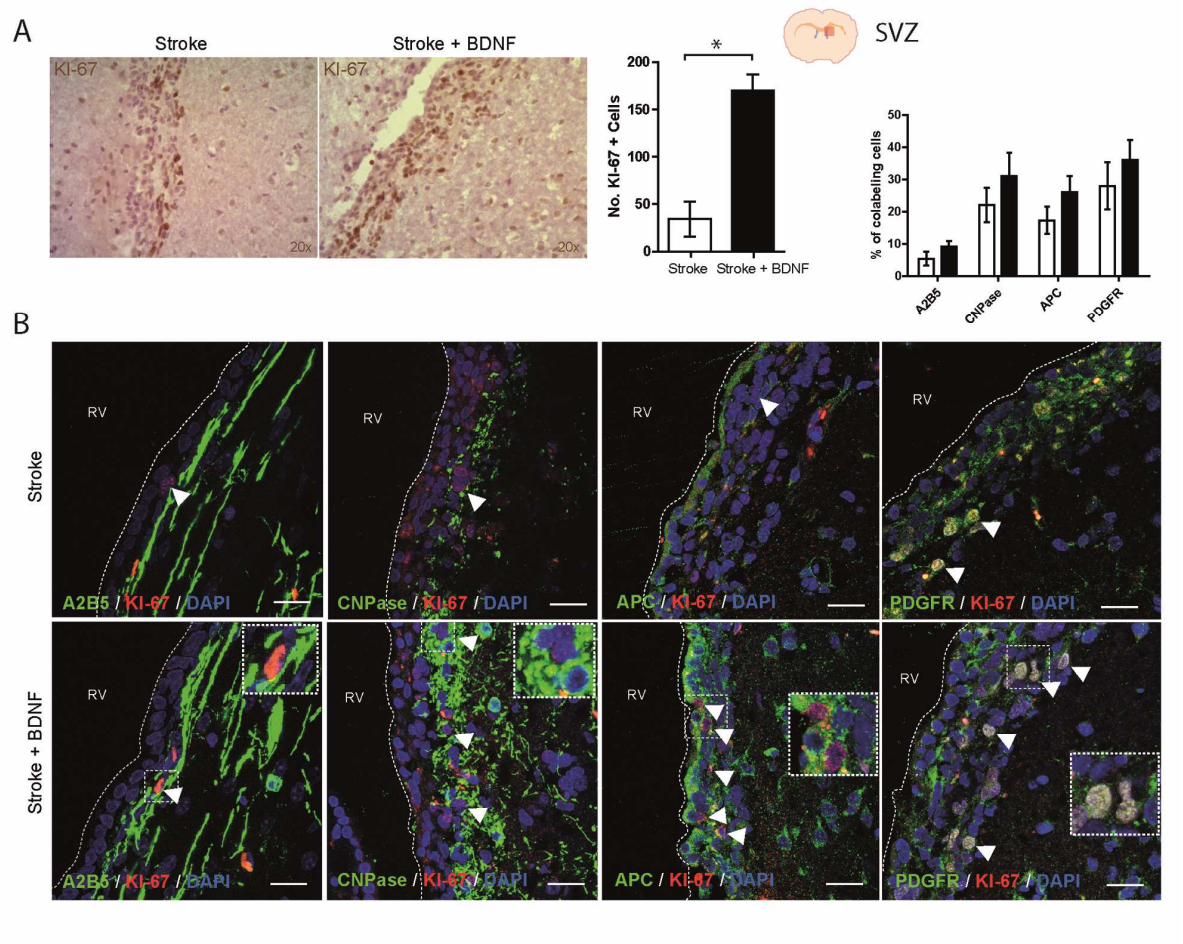

A
Stroke
Stroke + BDNF
KI-67
KI-67
20x
20x
SVZ
*
No. KI-67 + Cells
200
150
100
50
0
Stroke
Stroke + BDNF
% of colabeling cells
50
40
30
20
10
0
A2B5
CNPase
APC
PDGFR
B
Stroke
Stroke + BDNF
RV
A2B5 / KI-67 / DAPI
CNPase / KI-67 / DAPI
APC / KI-67 / DAPI
PDGFR / KI-67 / DAPI

Figura 4

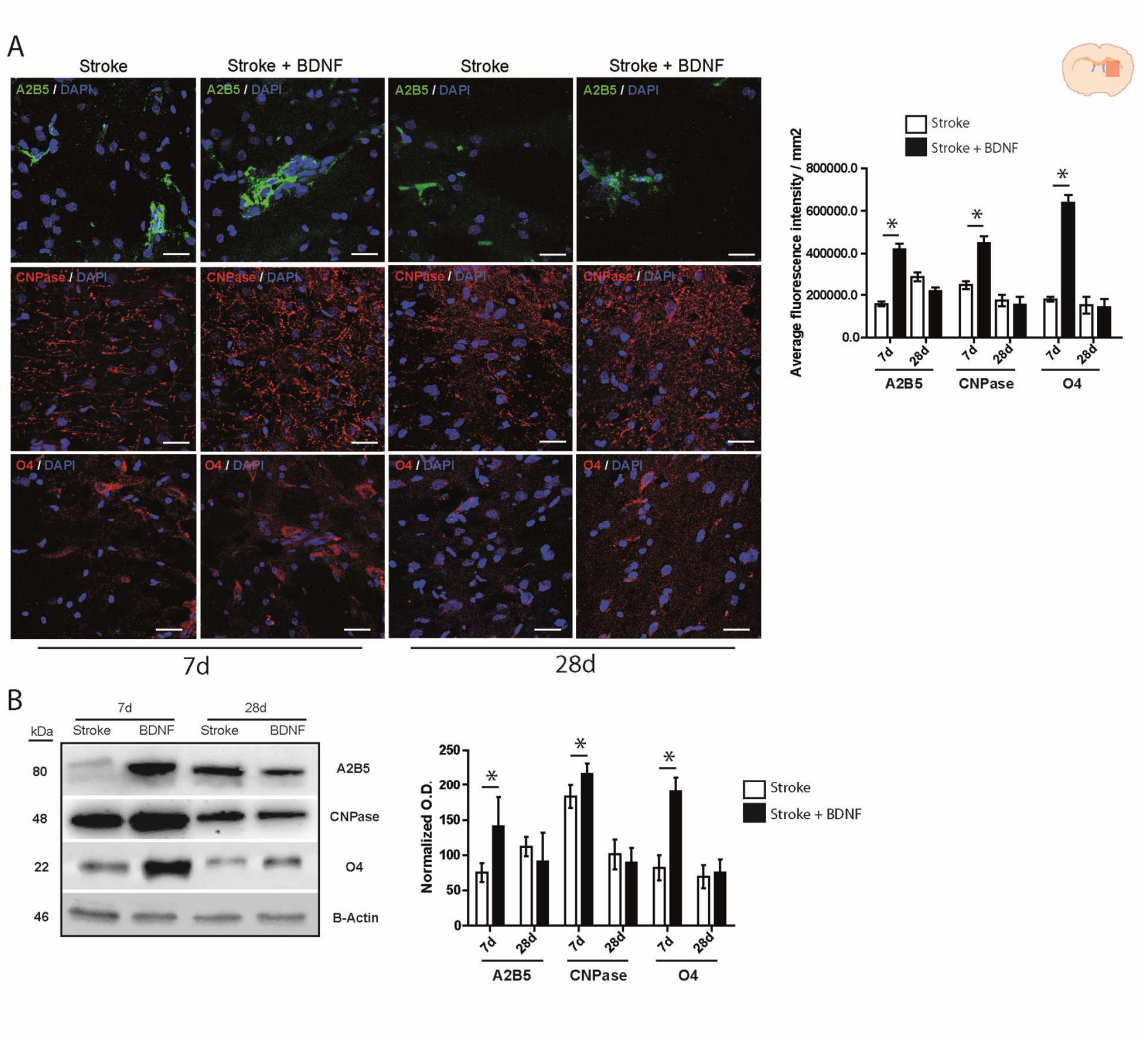
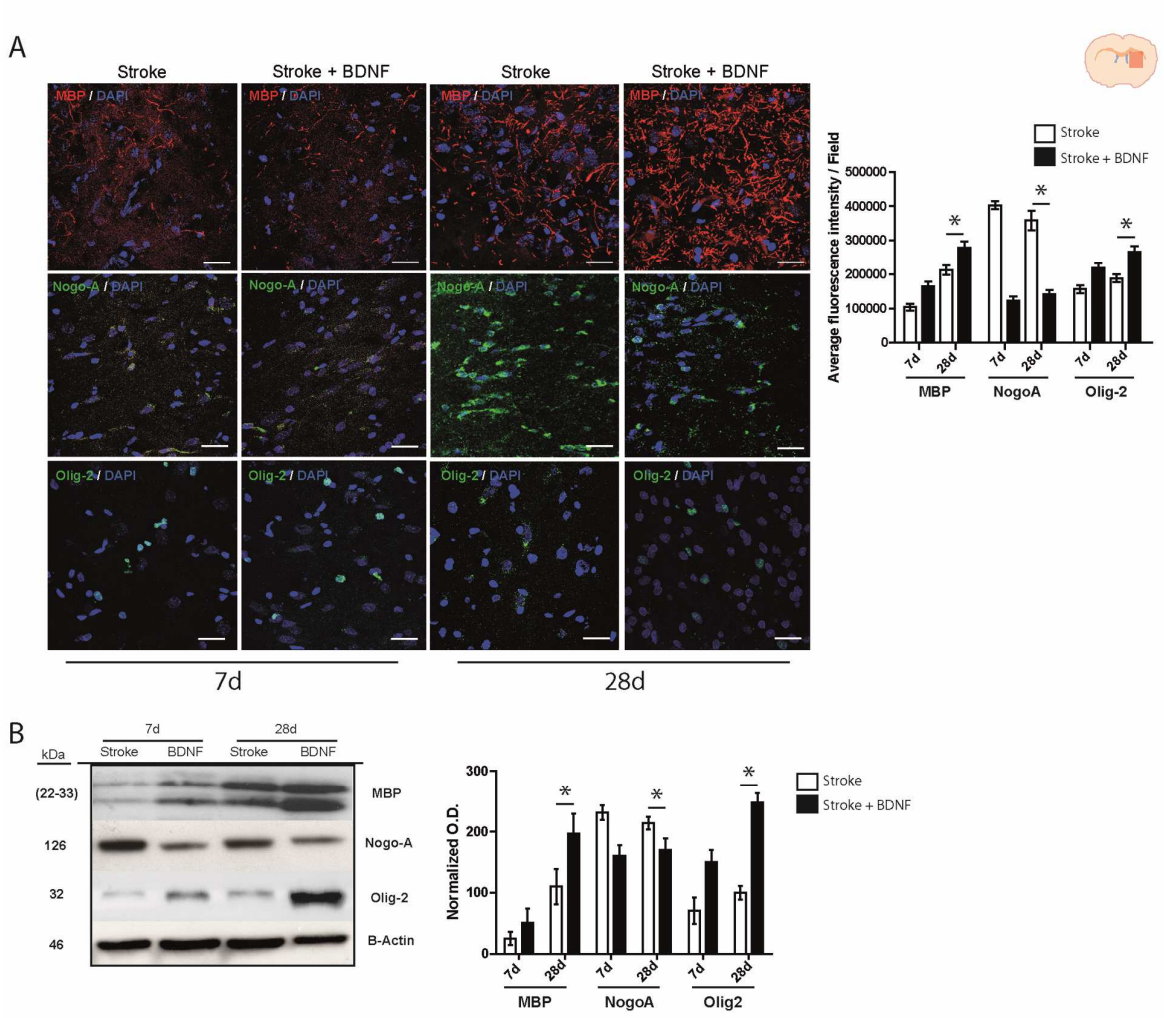

A
Stroke
Stroke + BDNF
Stroke
Stroke + BDNF
A2B5 / DAPI
CNPase / DAPI
O4 / DAPI
7d
28d
Stroke
Stroke + BDNF
Average fluorescence intensity / mm2
800000.0
600000.0
400000.0
200000.0
0.0
7d
28d
A2B5
CNPase
O4
B
kDa
7d
28d
Stroke
BDNF
Stroke
BDNF
80
48
22
46
A2B5
CNPase
O4
B-Actin
Normalized O.D.
250
200
150
100
50
0
Stroke
Stroke + BDNF

Figura 5


A
Stroke
Stroke + BDNF
Stroke
Stroke + BDNF
MBP / DAPI
Nogo-A / DAPI
Olig-2 / DAPI
7d
28d
Stroke
Stroke + BDNF
Average fluorescence intensity / Field
500000
400000
300000
200000
100000
0
7d
28d
MBP
NogoA
Olig-2
B
kDa
7d
28d
Stroke
BDNF
(22-33)
126
32
46
MBP
Nogo-A
Olig-2
B-Actin
Normalized O.D.
300
200
100
0
Olig2

Artículo número 3:

White Matter Injury Restoration after Stem Cell Administration in Subcortical Ischemic Stroke

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RESEARCH Open Access

White matter injury restoration after stem cell administration in subcortical ischemic stroke

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Abstract

Introduction: Despite its high incidence, nerve fiber (axon and myelin) damage after cerebral infarct has not yet been extensively investigated. The aim of this study was to investigate white matter repair after adipose-derived mesenchymal stem cell (ADMSC) administration in an experimental model of subcortical stroke. Furthermore, we aimed to analyze the ADMSC secretome and whether this could be implicated in this repair function.

Methods: An animal model of subcortical ischemic stroke with white matter affectation was induced in rats by injection of endothelin-1. At 24 hours, 2×10^6 ADMSC were administered intravenously to the treatment group. Functional evaluation, lesion size, fiber tract integrity, cell death, proliferation, white matter repair markers (Olig-2, NF, and MBP) and NogoA were all studied after sacrifice (7 days and 28 days). ADMSC migration and implantation in the brain as well as proteomics analysis and functions of the secretome were also analyzed.

Results: Neither ADMSC migration nor implantation to the brain was observed after ADMSC administration. In contrast, ADMSC implantation was detected in peripheral organs. The treatment group showed a smaller functional deficit, smaller lesion area, less cell death, more oligodendrocyte proliferation, more white matter connectivity and higher amounts of myelin formation. The treated animals also showed higher levels of white matter-associated markers in the injured area than the control group. Proteomics analysis of the ADMSC secretome identified 2,416 proteins, not all of them previously described to be involved in brain plasticity.

Conclusions: White matter integrity in subcortical stroke is in part restored by ADMSC treatment; this is mediated by repair molecular factors implicated in axonal sprouting, remyelination and oligodendrogenesis. These findings are associated with improved functional recovery after stroke.

Introduction

White matter injury and the mechanisms of nerve fiber (axon and myelin) repair have seldom been investigated in translational stroke research [1, 2], despite the fact that blood supply disruption also compromises whole axons and fibers and therefore brain connectivity. Even though relevant, white matter injury in stroke has not been extensively studied in the past due to the intrinsic difficulties associated with animal models; for instance, the fact that the rodent brain has substantially less white matter than higher mammals or humans [3, 4]. However, not only are up to 25 % of ischemic strokes in humans subcortical [1], but cortical infarcts also produce white matter injury. The high incidence of such damage motivates the search for an effective therapy that would enhance the mechanisms underlying the repair of damaged nerve fibers after any kind of stroke.

Stem cell therapy has demonstrated its efficacy in cortical stroke and may have a positive effect on subcortical lesions. In this regard, preclinical studies indicate that adipose-derived mesenchymal stem cells (ADMSC) are a promising new therapy for subcortical stroke that could promote recovery by improving the global brain repair mechanisms [5–7]. Trophic factor release, paracrine interactions and immunomodulatory effects have been

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suggested as the main functional mechanisms involved in ADMSC therapy [8, 9]. In this regard, stem cells are known to have paracrine effects on neurogenesis, gliogenesis, synaptogenesis, vasculogenesis and immunomodulation. However, there is little evidence whether stem cell administration can promote oligodendrogenesis and white matter fiber repair when axonal tract integrity has been compromised.

Therefore, the aim of this study was to investigate the therapeutic effects (improvement of functional deficits and enhancement of white matter fiber repair) of the intravenous administration of ADMSC in rats submitted to subcortical stroke with white matter injury.

Methods

Ethics statement

The procedure was carried out at our Cerebrovascular and Neuroscience Research Laboratory, La Paz University Hospital, Madrid, Spain. All experiments were designed to minimize animal suffering in compliance with, and approved by, our medical school's Ethical Committee of La Paz University Hospital for the Care and Use of Animals in Research according to the Spanish and European Union rules (86/609/CEE, 2003/65/CE, 2010/63/EU, RD 1201/2005 and RD53/2013).

Animals and surgery

A total of 72 male Sprague–Dawley rats weighing 200–250 g (Charles River Laboratories, France) were used. In all animals, the femoral artery was cannulated during surgery and induction of cerebral ischemia to allow continuous monitoring of physiological parameters including blood glucose levels, blood gases and blood pressure (Omicron ALTEA Monitor; RGB Medical Devices, Madrid, Spain). Cranial and body temperature were monitored and maintained at 36.5 ± 0.5 °C. Male Sprague–Dawley rats (200–250 g) were anesthetized using 3.5 % isoflurane in 2 L/minute oxygen and given meloxicam 2 mg/kg for analgesia. To provoke white matter injury, subcortical stroke was induced by injection of 1 μL endothelin-1 (ET-1; Calbiochem, Germany) (0.25 μg/μL) with the use of a SYR 5 μL Hamilton syringe (Tecknokroma, Barcelona, Spain) into the striatum using stereotactic references (+0.4 mm AP, +3.5 mm L, + 6 mm DV from bregma) as previously described [10].

After 24 hours, the treatment group received intravenously (i.v.) 2×10^6 ADMSC in 1 ml of saline solution (n = 24) by the tail vein. Dose was determined based on previous studies [8, 9]. In the control (n = 24) and sham-operation (n = 24) groups, only saline solution was i.v. administered via the tail vein. Rats were sacrificed at 24 hours (n = 4 in each group for comparative anatomical analysis of the lesion in fresh tissue and to analyze ADMSC distribution), 7 days (n = 10 in each group) or 28 days (n = 10 in each group) after treatment for cell death and proliferation analysis and immunohistochemistry, immunofluorescence and Western blot studies.

Cell culture protocol

ADMSC obtained from allogeneic adipose tissue of Sprague–Dawley rats (250–300 g) were cultured. The adipose tissue was digested with collagenase (Sigma Aldrich, Madrid, Spain) and incubated at 37 °C in 5 % CO_2. On the third pass, the cell cultures were divided into three groups: 1) 1.0×10^5 ADMSC for characterization, 2) 1.5×10^6 ADMSC for proteomics analysis of the culture supernatant, and 3) 42×10^6 ADMSC for the treatment of rats. For characterization, ADMSC were trypsinized and labeled with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or Alexa 647-conjugated primary antibodies. The cells were incubated for 20 minutes at 4 °C in the dark with the following antibodies: CD90-FITC (AbD Serotec, Oxford, UK), CD29-PE (AbD Serotec), CD45-PE (AbD Serotec) and CD11b-PE (AbD Serotec). Matched isotype controls were purchased from Biolegend (San Diego, CA, USA). Flow cytometry analysis of CD90+/CD29+/CD45−/CD11b− cells was performed using a FACScalibur cytometer and CellQuest Pro software (Becton Dickinson, Madrid, Spain). For ADMSC treatment, ADMSC with >95 % viability were administered i.v. The dose, route and time of administration were based on previously reported data [9, 11].

Proteomics data analysis

For proteomic analysis of cell culture supernants, ADMSC were cultured overnight with a free fetal bovine serum/protein culture medium. After 24 hours, cell supernatants were collected and the protein content was analyzed as follows. Proteins were digested using the filter aided sample preparation (FASP) protocol [12]. Briefly, samples were dissolved in 50 mM Tris–HCl pH 8.5, 4 % SDS and 50 mM DTT, boiled for 10 minutes and then centrifuged. Protein concentration in the supernatant was measured by the Direct Detect® Spectrometer (Millipore, Billerica, MA, USA). Approximately 50 μg of protein was diluted in 8 M urea in 0.1 M Tris–HCl (pH 8.5), and loaded onto 30 kDa centrifugal filter devices (FASP Protein Digestion Kit, Expedeon, Knoxville, TN, USA). The denaturation buffer was replaced by washing three times with UA. Proteins were later alkylated using 50 mM iodoacetamide in UA for 20 minutes in the dark, and the excess alkylation reagents were eliminated by washing three times with UA and three additional times with 50 mM ammonium bicarbonate. Proteins were digested overnight at 37 °C with modified trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate at 40:1 protein to trypsin (w/w) ratio. The resulting peptides were eluted by centrifugation with 50 mM

ammonium bicarbonate (twice) and 0.5 M sodium chloride. Trifluoroacetic acid (TFA) was added to a final concentration of 1 % and the peptides were finally desalted onto C18 Oasis-HLB cartridges and dried-down for further analysis.

Peptides were loaded into the LC-MS/MS liquid chromatography tandem mass spectrometry system for on-line desalting onto C18 cartridges and analyzing by LC-MS/MS using a C-18 reversed phase nano-column (75 μm internal diameter × 50 cm, 2 μm particle size, Acclaim PepMap RSLC, 100 C18; Thermo Fisher Scientific, Waltham, MA, USA) in a continuous acetonitrile gradient consisting of 0–30 % B in 180 minutes, 50–90 % B in 3 minutes (B = 90 % acetonitrile, 0.5 % formic acid). A flow rate of 200 nL/minute was used to elute peptides from the RP nano-column to an emitter nanospray needle for real time ionization and peptide fragmentation on an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). An enhanced FT-resolution Fourier- Transform spectrum (resolution = 35,000) followed by the MS/MS spectra from the most intense 15 parent ions were analyzed along the chromatographic run. Dynamic exclusion was set at 30 seconds.

For peptide identification, all spectra were analyzed with Proteome Discoverer (version 1.4.0.29; Thermo Fisher Scientific) using SEQUEST-HT (Thermo Fisher Scientific). For database searching on the Uniprot database containing all sequences from humans (6 March 2013), parameters were selected as follows: trypsin digestion with two maximum missed cleavage sites, precursor and fragment mass tolerances of 600 ppm and 0.02 Da, respectively, carbamidomethyl cysteine as fixed modification and methionine oxidation as dynamic modifications. Peptide identification was validated using the probability ratio method [13] with an additional filtering for precursor mass tolerance of 10 ppm. False discovery rate (FDR) was calculated using inverted databases and the refined method [14]. For the study of the biological functions of identified proteins, gene onthology analysis was performed using the GOrilla (Gene Ontology Enrichment Analysis and Visualization) research tool [15].

Biodistribution analysis

For identification of donor cells, ADMSC were labeled with DiI (Celltracker CM-DiI, Invitrogen, Barcelona, Spain) prior to administration and stained with CD90, and possible migration and implantation were analyzed using immunofluorescence. Biodistribution of labeled ADMSC with DiI 24 hours after i.v. administration was analyzed using immunofluorescence techniques in both control and treated animals. Cryosections (10 μm thick) of brain, kidney, liver, lung and spleen were counterstained with 4′,6-diamino-2-phenylindole (DAPI) and analyzed by immunofluorescence staining (n = 4 per group).

Functional evaluation

Functional evaluation was performed in all animals by a blinded observer before surgery and after 1, 3, 7, 14 and 28 days. Motor performance was evaluated using the beam walking and rotarod tests and Rogers' functional scale. The beam walking test measured the ability of rats to walk along a wooden beam (2.5 × 2.5 × 80 cm). Scores were assigned as follows: 0, traversed the beam with no foot slip; 1, traversed with grasping of the lateral side of the beam; 2, difficulty crawling along the beam but able to traverse; 3, required >10 seconds to traverse the beam because of difficulty with walking; 4, unable to traverse the beam; 5, unable to move the body or any limb on the beam; and 6, unable to stay on the beam for >10 seconds [16]. The rotarod test measured the latency to fall from a rotating cylinder [9]. A variant of Rogers' functional scale was used to assign scores as follows: 0, no functional deficit; 1, failure to extend forepaw fully; 2, decreased grip of forelimb while tail gently pulled; 3, spontaneous movement in all directions, contralateral circling only if pulled by the tail; 4, circling; 5, walking only when stimulated; 6, unresponsive to stimulation with a depressed level of consciousness; and 7, dead (n = 10 per group) [17].

In vivo magnetic resonance imaging and tractography

Lesion size was analyzed after 1, 7 and 28 days by magnetic resonance imaging (MRI) using a 7-Tesla horizontal bore magnet (Bruker Pharmascan, Ettlingen, Germany) and T2 maps (RARE 8 T2, 180° flip angle, three averages) as previously described [9]. The lesion area was expressed as a percentage of the contralateral hemisphere, after correcting for brain edema. For tractography, diffusion tensor imaging (DTI) was performed after 1, 7 and 28 days using a spin-echo single-shot echo-planar imaging pulse sequence with the following parameters: TR/TE, 5000/35 ms; signal average, 10; 30 non-collinear diffusion gradients with diffusion weighting of b = 1,000 s/mm^2 and b = 0 s/mm^2; and field of view 3.5 × 3.5 cm. A total of 496 slices were evaluated from data acquired in 30 directions. The images were obtained using medInria (Inria, France), a multi-platform medical image processing and visualization software. Zoomed lesion site three-dimensional diffusion tensor images were represented using ParaView 4.1.0 software (Los Álamos National Laboratory, New México, USA) (n = 6 per group, 10 sections per animal) as previously described [10].

Cell death evaluation

Cell death was analyzed in the infarct zone of at least 10 sections from each animal using TUNEL staining (TdT-FragEL DNA Fragmentation Detection Kit, Oncogene Research Products, San Diego, CA). The number of positive cells was counted in a minimum of 10 different

microscopical fields based on their nuclear morphology and dark color using a 40× objective lens and image analysis software (Image-Pro Plus 4.1, Media Cybernetics, Rockville, MD, USA) (n = 6 per group, 10 sections per animal).

Cell proliferation analysis

Cell proliferation was analyzed using Ki-67 staining (1:100, Chemicon, Temecula, CA, USA) after 7 and 28 days, in 10 sections corresponding to the infarct area of each animal, selected as previously described [18, 19]. The number of positive cells was counted in a minimum of 10 different random microscopic fields using a 40× objective lens and Image-Pro Plus 4.1 software (n = 6 per group, 10 sections per animal).

The differentiation of the proliferating cells was analyzed using co-staining with Ki-67 and NeuN (1:100, Millipore), Olig-2 (1:400, Millipore) and glial fibrillary acidic protein (GFAP; 1:400, Chemicon) followed by goat anti-mouse Alexa Fluor 488 antibody (1:750, Invitrogen). Images were acquired as a confocal maximum projection using a Leica TCS-SPE confocal microscope (Leica Microsystems, Heidelberg, Germany) and the number of double-positive cells was counted in a minimum of 10 different microscopic fields using a 40× objective lens and Image-Pro Plus 4.1 software.

Immunohistochemical, immunofluorescence and Western blot analyses

Frozen sections were stained using the CryoMyelin Kit (Hitobiotech, Wilmington, USA), which allows sensitive localization and visualization of myelin fibers. The mean intensity of myelin staining in the region of interest (ROI) was quantified using a Nikon Eclipse-Ti inverted microscope and NIS-elements software. The lesion area was studied in detail using immunofluorescence and Western blot analyses. The white matter-associated antibodies used were neurofilament (NF; 1:100, Dako, Glostrup, Denmark), neurite outgrowth inhibitor (NogoA; 1:100, Abcam, Cambridge, UK), myelin basic protein (MBP; 1:100, Abcam) and oligodendrocyte (Olig-2; 1:500, Millipore) followed by goat anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 (1:750, Invitrogen). For Western blot analysis, the units were normalized based on B-actine (1:400, Sigma-Aldrich). To quantify the expression of white matter-associated markers, the mean fluorescence intensity was evaluated in a minimum of 10 different microscopic fields using a 40× objective lens. The experiments, images and quantification of the samples were performed on the same day using the same microscope configurations, by blinded observers, to eliminate bias due to background normalization (four animals, four sections per animal).

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Data were compared using the Kruskal-Wallis test followed by the Mann–Whitney test. Values of $p < 0.05$ were considered as statistically significant. The analysis was performed using statistical SPSS 16 and GraphPad software (GraphPad Software Inc, CA, USA).

Results

ADMSC characterization, migration and implantation in the injured brain area

ADMSC showed typical fibroblast-like cell morphology and their phenotype was CD90+/CD29+/CD45−/CD11b− (Fig. 1a).

DiI and CD90 co-labeled cells were not observed in the control group. Migration and implantation in the brain were not observed on immunofluorescence images of the injured brain area after intravenous administration of DiI and CD90 co-labeled cells. However, DiI and CD90 co-labeled cells were observed in peripheral organs such as the liver, lung and spleen (Fig. 1b).

Effect of ADMSC treatment on functional recovery

To assess the potential of ADMSC administration to improve functional recovery after subcortical stroke, motor function was assessed before surgery and after 1, 3, 7, 14 and 28 days using the walking beam, rotarod and Roger's test. There were significant differences in motor deficit scores between the treatment and control group; the walking beam test performance was significantly better in the treatment group than in the control group after 3 days ($p < 0.01$), 14 days ($p < 0.05$) and 28 days ($p < 0.05$). The Rotarod test was significantly better in the ADMSC treatment group than in the control group after 1 day ($p < 0.01$) and 28 days ($p < 0.05$). The Rogers' functional scale score was significantly better in the treatment group than in the control group after 7 and 28 days (both $p < 0.05$) (Fig. 2).

Effect of ADMSC treatment on lesion size and tract connectivity

MRI analysis showed no significant difference in infarct size between the treatment and control groups after 1 and 7 days. However, the infarct size was significantly smaller in the treatment group than in the control group after 28 days (0.12 ± 0.01 vs. 0.6 ± 0.26, $p < 0.05$) (Fig. 3).

Myelin was stained using the CryoMyelin Kit to identify the area of white matter injury in the subcortical infarct. The mean intensity of staining in the ROI was evaluated in the treatment and control groups, with white indicating absence of myelin and black indicating the presence of myelinated axons. There was higher intensity (indicating absence of myelin) in the control

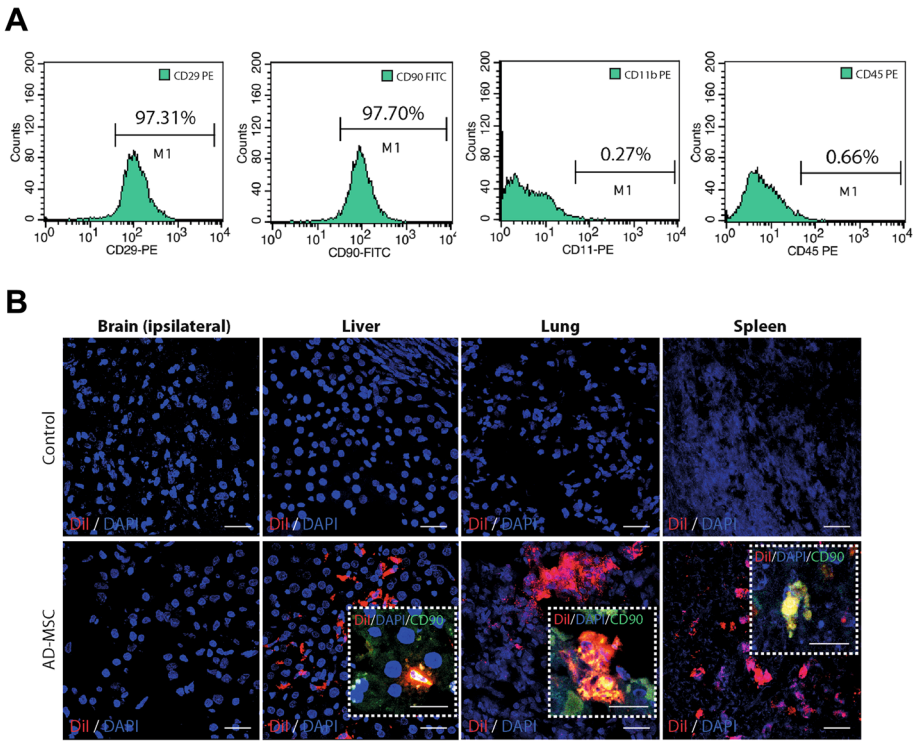


Fig. 1 Characterization and biodistribution of ADMSC. **a** ADMSC characterization by flow cytometry. Rat ADMSC were labeled with CD29, CD90, CD11b, and CD45 and analyzed by flow cytometry. Of the ADMSC population, 95 % expressed CD29 and CD90. Additionally, these cells lacked expression (5 % positive) of CD11b, CD45. **b** Migration and implantation in the brain and peripheral organs (liver, lung and spleen) of DiI- and CD90-labelled cells at 24 hours after treatment. *AD-MSC* adipose-derived mesenchymal stem cells, *DAPI* 4′,6-diamino-2-phenylindole, *FITC* fluorescein isothiocyanate, *PE* phycoerythrin

group (210.23 ± 14.30 mean intensity) than in the treatment group (155.71 mean intensity ± 21.23) (Fig. 3).

DTI tractography data showed similar results in axial diffusivity (120.35 ± 12.45 $\mu m^2/s$ and 126.78 ± 18.34; $p > 0.05$) in both the control and treated groups, respectively, at 7 days. However, compared with the control rats, 28 days after treatment the ADMSC-treated rats showed significantly improved axial diffusivity (127.98 ± 9.21 and 162.99 ± 13.65 $\mu m^2/s$, respectively; $p < 0.05$) compared to controls. These results suggest that there was a significant improvement in white matter thickness (width, breadth, depth) and restoration of tract connectivity in the ADMSC-treated animals compared with controls at 28 days.

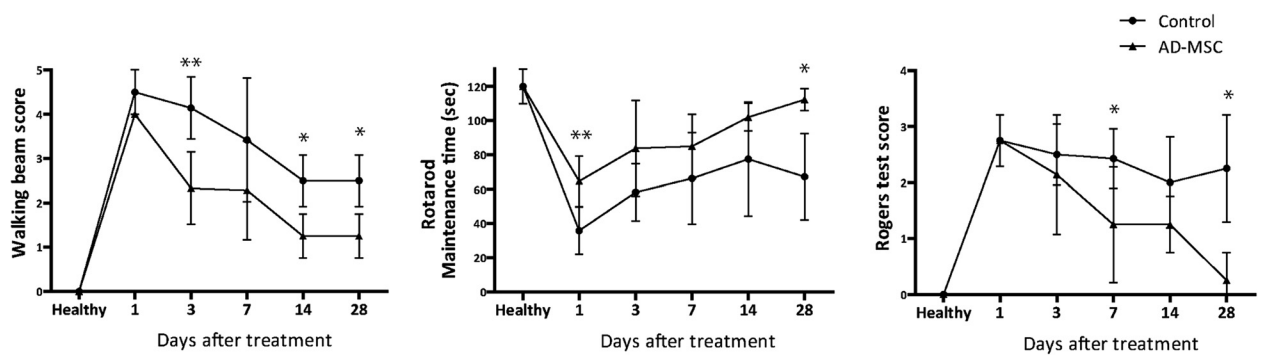


Fig. 2 Improved functional outcome after ADMSC administration in subcortical stroke. Beam walking test performance (*left*) was improved at 3 ($p < 0.01$), 14 and 28 days ($p < 0.05$). Rotarod test (*middle*) showed significant differences between the ADMSC group and control animals at 1 ($p < 0.01$) and 28 days ($p < 0.05$). The ADMSC group also showed significantly better scores compared to controls at 7 and 28 days ($p < 0.05$) on the Roger's test (*right*). Data are shown as mean ± SEM; *$p < 0.05$, **$p < 0.005$; n = 10 animals per group. *AD-MSC* adipose-derived mesenchymal stem cells

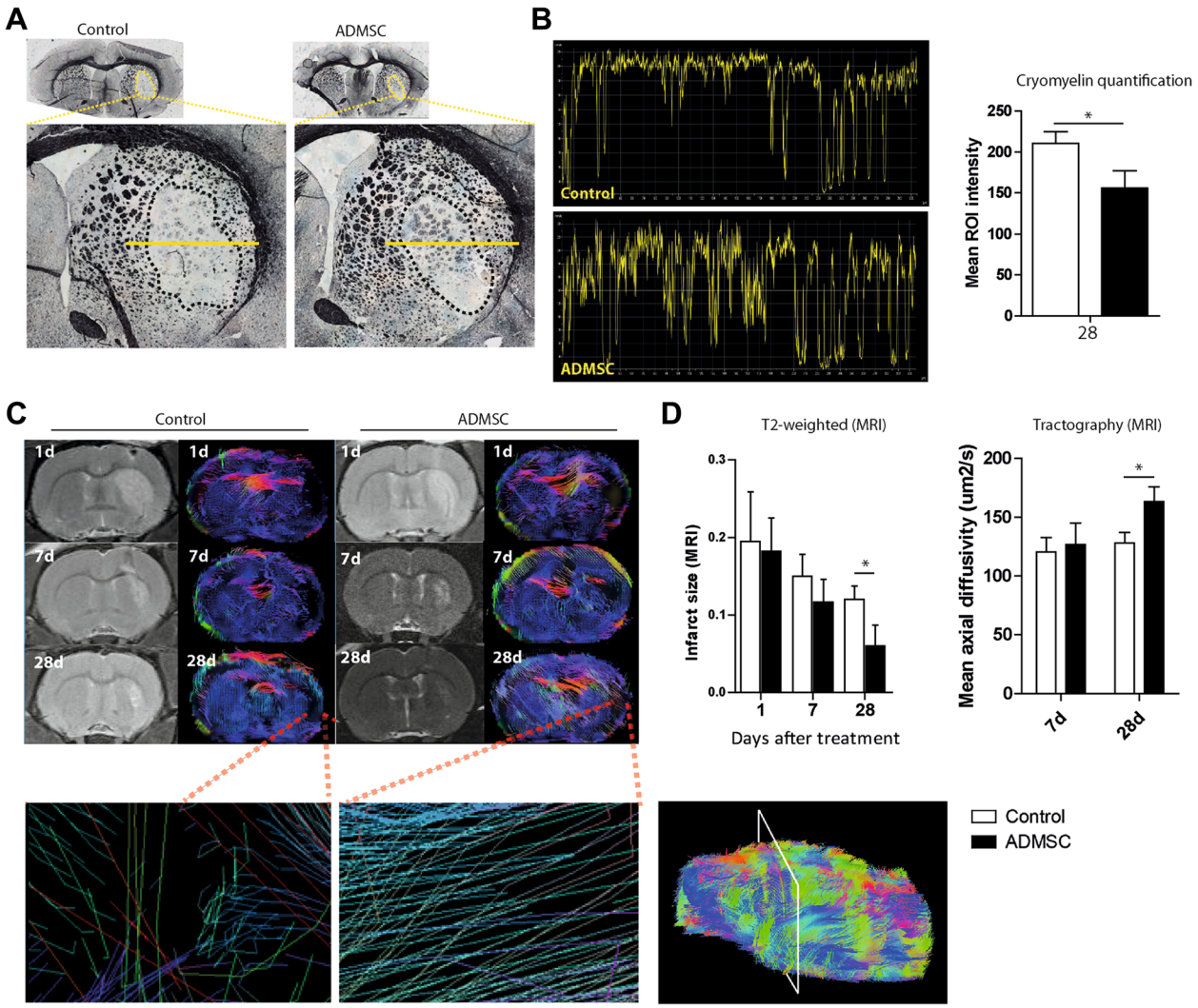


Fig. 3 ADMSC treatment reduced infarct size and increased fiber tract and myelin integrity after subcortical white matter damage. **a** Morphological study by CryoMyelin staining identified the zone of the lesion as an area of white matter injury located in the subcortical zone, showing restored myelinated axons in the ADMSC-treated animals. **b** Quantification of mean ROI intensity of the CryoMyelin staining. Stroked line indicates ROI; yellow line indicates a representative longitudinal profile of pixel intensity. **c** Comparative image analysis T2-weighted MRI and tractography at 1, 7 and 28 days showed a progressive reduction in white matter infarct size in controls and treated animals. Detail of tractography image in the lesion is given below showing augmented connectivity of fiber tracts in ADMSC-treated animals at 28 days. **d** Quantitative analysis of MRI images showed that ADMSC therapy reduced lesion size at 28 days compared to the controls ($p < 0.05$). Data are shown as mean ± SEM; *$p < 0.05$; n = 6 animals, 10 sections each per group. *ADMSC* adipose-derived mesenchymal stem cells, *d* days, *MRI* magnetic resonance image, *ROI* region of interest

Effect of acute ADMSC treatment on cell death and brain cell proliferation

Cell death was analyzed on frozen sections by TUNEL staining after 7 and 28 days. After 7 days, no significant differences were found in TUNEL-positive cells in both the control group (718.5 ± 146.3 cells) and the treatment group (460.33 ± 120.5 cells). After 28 days, there were significantly fewer TUNEL-positive cells in the ischemic area in the treatment group than in the control group (24.5 ± 1.73 vs. 56.0 ± 3.46 cells, $p < 0.05$) (Fig. 4a).

Quantitative analysis of proliferative cells was performed using Ki-67 labeling after 7 and 28 days. After 7 days, there were no significant differences in proliferative cells in both the treatment group (83.5 ± 34.65 cells) and the control group (19.5 ± 7.78 cells) (Fig. 4b). After 28 days, the number of Ki-67-positive cells was significantly higher in the treatment group than in the control group (13 ± 1.15 vs. 3 ± 1.15 cells, $p < 0.05$). The proportions of proliferating cell types observed by co-staining with Ki-67 were not significantly different

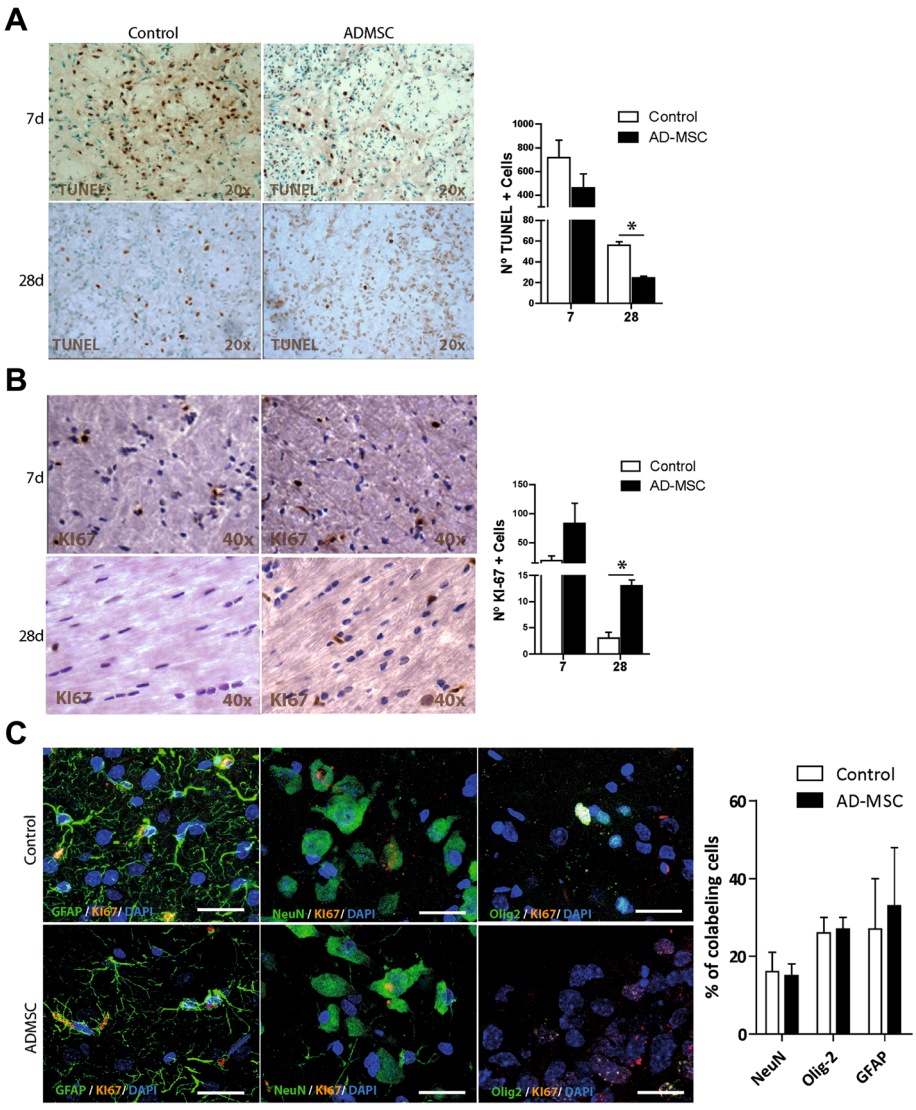


Fig. 4 ADMSC administration led to a cell death reduction and improved brain proliferation activity. **a** Quantitative analysis of cell death by TUNEL technique showed a significant reduction in TUNEL-positive cells after ADMSC therapy compared to the control group ($p < 0.05$). **b** At 28 days, Ki-67 staining shows a significant increase in the number of proliferating cells in ADMSC-treated animals compared to the control group ($p < 0.05$). **c** At 28 days after treatment, Ki-67 co-labeling with NeuN, GFAP, and Olig-2 showed different cell type proliferation including oligodendrocytes. Data are shown as mean ± SEM; scale bars = 20 μm; n = 6 animals, 10 sections each per group. *ADMSC* adipose-derived mesenchymal stem cells, *DAPI* 4′,6-diamino-2-phenylindole, *GFAP* glial fibrillary acid protein, *NeuN* neuronal nuclei, *Olig-2* oligodendrocite transcription factor 2

between the treatment and control groups (NeuN, 15 ± 3 % vs. 16 ± 5 %, Olig-2: 27 ± 3 % vs. 26 ± 4 %; GFAP, 33 ± 15 % vs. 27 ± 13 %) (Fig. 4c). However, although there were no significant differences between the proportions of the cell lines, higher levels of each cell type were found in the ADMSC-treated group.

Effect of ADMSC treatment on white matter-associated marker expression

After finding that ADMSC administration had a beneficial effect on functional outcome, we investigated whether the functional outcome was related to the levels of white matter-associated markers. Western blot analysis found that the level of NF (a marker of axonal sprouting) was not significantly different between the treatment and control groups after 7 days. However, the NF level was significantly higher in the treatment group than in the control group after 28 days (4.27 ± 1.26 vs. 1.00 ± 0.69 Arbitrary Units (AU), $p < 0.001$). The MBP level was significantly higher in the treatment group than in the control group after 7 days (1.09 ± 0.23 vs. 0.63 ± 0.18 AU, $p < 0.05$) and 28 days (0.80 ± 0.11 vs.

0.43 ± 0.24 AU, $p < 0.05$). The Olig-2 level was significantly higher in the treatment group than in the control group after 28 days (1.08 ± 0.10 vs. 0.32 ± 0.08 AU, $p < 0.05$). The NogoA level was not significantly different between the treatment and control groups after 7 days (0.68 ± 0.35 vs. 0.67 ± 0.17 AU, $p > 0.05$), and tended to be lower in the treatment group than in the control group after 28 days (0.55 ± 0.35 vs. 1.16 ± 0.34 AU, $p > 0.05$) (Fig. 5). The NF immunofluorescence intensity was significantly higher in the treatment group than in the control group after 28 days (10,020.28 ± 1,231.19 vs. 3,536.21 ± 643.2 average fluorescence intensity, $p < 0.05$). The MBP immunofluorescence intensity was significantly higher in the treatment group than in the control group after 7 days (5,714.61 ± 529.59 vs. 3,529.97 ± 1,222.40 AU, $p < 0.05$) and 28 days (6,920.39 ± 1,134.27 vs. 3,736.34 ± 324.50 AU, $p < 0.05$). The Olig-2 immunofluorescence intensity was significantly higher in the treatment group than in the control group after 28 days (2,439.00 ± 231.12 vs. 353.40 ± 111.12 AU, $p < 0.05$). The NogoA immunofluorescence intensity tended to be lower in the treatment group

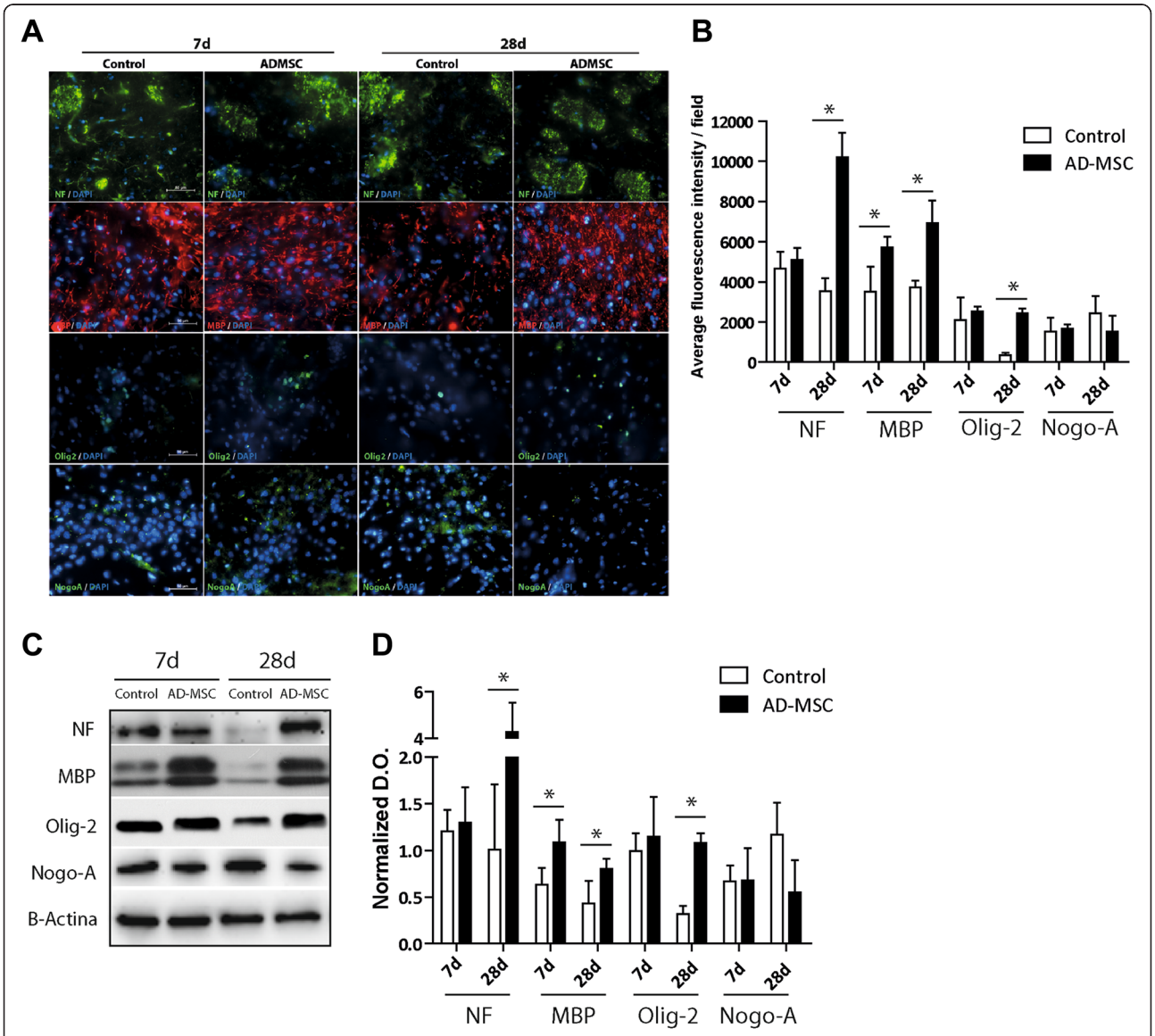
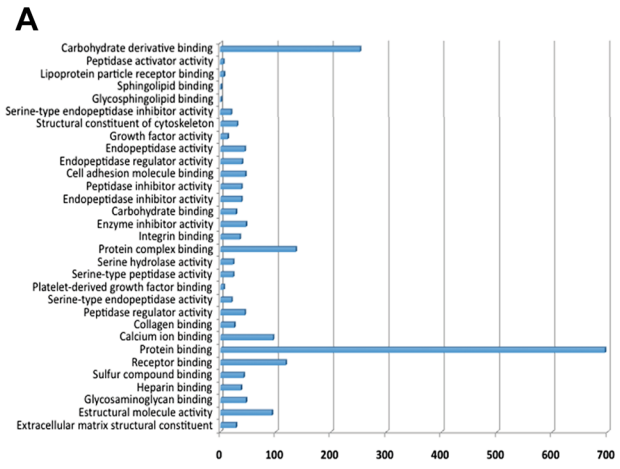
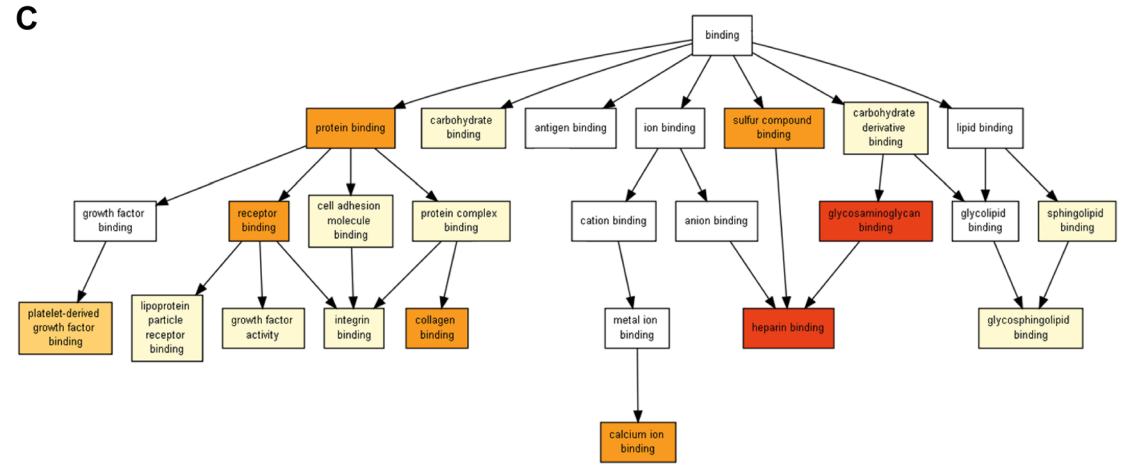


Fig. 5 White matter-associated markers are enhanced in striatum after ADMSC therapy in subcortical stroke model. **a** Immunofluorescence images and **b** immunofluorescence quantification of white matter repair-associated markers (NF, MBP, Olig-2 and NogoA) at 7 and 28 days after treatment. **c** Western blot and **d** Western blot quantification showed increased levels of MBP in the treated group compared to controls at both 7 and 28 days ($p < 0.05$), as well as augmented levels of Olig-2, NF ($p < 0.05$) and a trend to decreased levels of NogoA. Data are shown as mean ± SEM; scale bars = 20 μm; *$p < 0.05$; n = 4 animals, 4 sections each per group. *ADMSC* adipose-derived mesenchymal stem cells, *d* days, *MBP* myelin basic protein, *NF* neurofilament, *Nogo-A* neurite outgrowth inhibitor, *Olig-2* oligodendrocite transcription factor 2



B

Protein names	Full name	Code	FASTA	PSM
TGFBI	Transforming growth factor beta-induced	D4A8G5_RAT	D4A8G5	28.7
TGFB1	Transforming growth factor beta-1	TGFB1_RAT	P17246	2
TGFB2	Transforming growth factor beta-2	TGFB2_RAT	Q07257	2
VEGFC	Vascular endothelial growth factor C	VEGFC_RAT	O35757	2
VGFR2	Vascular endothelial growth factor receptor 2	VGFR2_RAT	O08775	1
CTGF	Conective tissue growth factor	CTGF_RAT	Q9R1E9	4
BDNF	Brain derived neurotrophic factor	BDNF_RAT	P23363	1
IGF2	Insulin-like growth factor II	IGF2_RAT	P01346	1
IBP2	Insulin-like growth factor binding protein 2	IBP2_RAT	P12843	1.33
IGF1R	Insulin-like growth factor 1 receptor	IGF1R_RAT	P24062	1
ALS	Insulin-like growth factor-binding protein complex acid labile subunit	ALS_RAT	P35859	1
HDGR2	Hepatoma-derived growth factor-related protein 2	HDGR2_RAT	Q925G1	2
Q5EBA7	Hepatocyte growth factor activator	Q5EBA7_RAT	Q5EBA7	1
LTBP1	Latent-transforming growth factor beta-binding protein 1	LTBP1_RAT	Q00918	7
LTBP2	Latent-transforming growth factor beta-binding protein 2	LTBP2_RAT	O35806	23.3



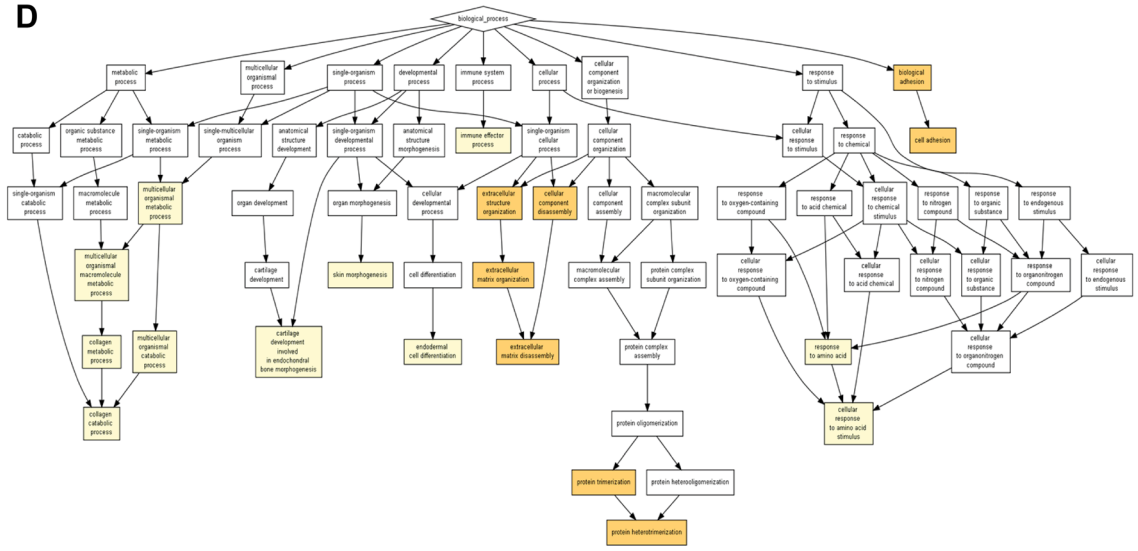


Fig. 6 Proteomics analysis of ADMSC secretome reveals multiple biological functions. **a** Gene onthology (GO) analysis of the 2,416 proteins identified by Orbitrap proteomic study. Bars indicate the number of proteins from the total associated with each biological function. **b** Trophic factor-related proteins identified in the proteomic analysis; PSM: peptide- spectrum matches **c** GO functional clustering of proteins associated with binding function and **d** other biological processes

than in the control group after 28 days (1,534.21 ± 767.32 vs. 2,423.88 ± 876.70 AU, $p > 0.05$).

Proteomics analysis of the ADMSC in vitro secretome

Proteomics analysis of the secretome of cell cultures identified 2,416 proteins in the cell supernatants that are implicated into different cell functions (Fig. 6a), such as protein binding (carbohydrate binding, antigen binding, ion binding, sulfur compound binding, and lipid binding), metabolic processes, single and multicellular organism processes, development, endodermal cell differentiation, skin and cartilage morphogenesis, immune system processes, cellular organization and biogenesis, response to stimulus and biological adhesion processes . All functions in detail are shown in Fig. 6c and d. Full proteomic data is given in Additional file 1. Interestingly, some of these proteins are also implicated in growth factor activity, such as several trophic factors and receptors known to be also involved in brain plasticity. Some of these trophic factors are shown in Fig. 6b.

Discussion

The results of this study showed that ADMSC administration plays a major role in improving the repair of white matter fiber tracts in an experimental model of subcortical stroke. We found that the treated group had better functional recovery and smaller lesion size than the control group. In addition, animals which received ADMSC treatment showed significantly higher number of proliferating cells (including oligodendrocyte progenitors) and significantly less cell death at the lesion region than animals in the control group. Analysis of fiber tract integrity by tractography and CryoMyelin staining showed that white tract thickness had been recovered in the treatment group. The treated group with ADMSC also had higher levels of white matter-associated markers (NF, MBP and Olig-2) than the control group, suggesting that ADMSC administration induced repair of white matter fiber tracts.

Up to 25 % of ischemic strokes in humans are subcortical or lacunar, which are confined to white matter regions such as the striatum and internal capsule [1]. The high frequency of damage to these areas in stroke patients has motivated the search for useful experimental animal models of subcortical stroke with white matter affectation, as well as effective therapies to enhance the mechanisms underlying repair of damaged white matter (axon and myelin). In this regard, endothelin (one of the most potent known vasoconstrictors) was considered the best candidate to induce this injury with white matter affectation [10, 20, 21].

ADMSC administration is considered an appropriate therapeutic strategy because ADMSC enhance the natural repair processes of the brain after injury. However, the mechanisms underlying these repair processes are still unknown. Our proteomics analysis identified thousands of proteins, many of them not previously associated with stem cell properties or stroke repair; for instance, we identified a number of proteins such as hepatoma-derived growth factor, latent-transforming growth factor beta, and connective tissue growth factor. Other proteins previously implicated in stem cell therapy function such as transforming growth factor-beta, fibroblast growth factor, vascular endothelial growth factor or brain-derived neurotrophic factor were also identified. Moreover, gene onthology analysis identified a number of protein functions not previously associated with stem cell therapy function in stroke recovery. In this regard, protein binding (carbohydrate binding, antigen binding, ion binding, sulfur compound binding, lipid binding), metabolic processes, single and multicelular organism processes, development, endodermal cell differentiation, skin and cartilage morphogenesis, immune system processes, cellular organization and biogenesis, response to stimulus and biological adhesion processes were highly represented. Interestingly, growth factor activity was not the main represented function in the cell secretome, indicating that other functions are also relevant. Our findings suggest that the release of the identified proteins by the administered ADMSC could contribute to improve functional recovery when allocated to peripheral organs (spleen, lung and liver). However, futures studies will be needed in order to understand the complex molecular mechanisms involved in stem cell therapy-mediated stroke recovery.

Various tests have been used to evaluate motor function following brain injury. Previous studies by our group have demonstrated improvement in the functional outcome after ADMSC administration in another experimental animal model of cerebral ischemia [9]. The present study showed that functional recovery at 28 days was significantly improved after ADMSC administration than in the control group after subcortical ischemic stroke.

In the present study, MRI studies showed a significantly smaller infarct size in the treated group than in the control group after 7 and 28 days. Our results are consistent with previous studies that reported a reduction in infarct size after ADMSC administration in another animal model of cerebral ischemia [9]. This reduction in infarct size could be related to the increased tract thickness and axonal projections observed by tractography and CryoMyelin staining. These results agree with previously reported findings that neural progenitor cell treatment in an animal model of cortical ischemia results in white matter reorganization shown by fiber tracking maps derived from DTI and by histological staining [22].

On the other hand, we found that the density of TUNEL-positive cells in the ischemic area peaked after 7 days in both treated and control groups. However, the treated group had less focal damage at 28 days, with significantly lower numbers of TUNEL-positive cells than in the control group. These results are consistent with those of previously reported studies, which found that ADMSC administration inhibited cell death in the infarct area [5, 9].

The central nervous system continuously generates new cells in several specific regions of the adult mammalian brain, and this proliferation has been shown to be enhanced by cell therapy. Ki-67 staining showed large numbers of proliferative cells after 7 days in both the treatment and control groups. In this regard, our results are consistent with a previous study [23]. Furthermore, there was greater cell proliferation after 28 days in the treated group than in the control group. These results provide clear evidence that ADMSC administration induces significant cell proliferation at 28 days after subcortical stroke. Furthermore, analysis of the proliferating cell lines showed similar proportions of co-staining with Ki-67 and NeuN, Olig-2 and GFAP in the treatment and control groups, indicating that almost half of the proliferating cells in both groups were white matter-associated cells (neurons and oligodendrocytes). These findings indicate genesis of new astrocytes and neurons as well as oligodendrocyte progenitors and immature oligodendrocytes after cerebral ischemia. Although the effects of ADMSC on neurogenesis, gliogenesis, synaptogenesis and vasculogenesis after stroke have already been described, the ability of ADMSC to promote oligodendrogenesis after subcortical stroke is a novel finding.

It is known that some brain repair mechanisms are quickly activated after cortical ischemia [24], but there is little information available regarding brain repair mechanisms after subcortical white matter stroke. To increase our understanding of these mechanisms, we investigated the levels of white matter-associated markers (NF, MBP, Olig-2 and NogoA) in both the treatment and control groups. NF levels, a marker of axonal sprouting, were not significantly different between the treated and control groups after 7 days, suggesting that the extent of white matter injury was similar in these groups during the acute phase. However, NF levels were higher in the group treated with ADMSC after 28 days, which could be explained by enhancement of axonal sprouting after ADMSC administration. In addition to axonal growth, restoration of the myelin sheath is important for the repair of white matter. Furthermore, Olig-2 levels were significantly higher in the treated group than in the control group after 28 days. These results support the concept that ADMSC enhance oligodendrogenesis to restore their loss due to ischemic injury. Our findings are supported by those of a recent in vitro study, which found that mesenchymal stem cell-conditioned medium promoted oligodendroglial cell maturation [25]. In addition to formation of mature oligodendrocytes, repair of the myelin sheath by oligodendrocytes is essential for achieving propagation of nerve impulses along axons [26]. The present study analyzed the MBP level as a marker of myelination. There were significantly higher levels of MBP in the treatment group compared to the control group after 7 and 28 days. These higher levels of MBP and Olig-2 are consistent with the increased proliferation observed for all phases of oligodendrocyte progenitors. Our results suggest that ADMSC administration increases oligodendrogenesis after white matter stroke.

Finally, among the myelin-associated proteins, NogoA, myelin-associated glycoprotein and oligodendrocyte myelin-associated protein all share a single receptor complex [27]. The present study did not find a difference in the NogoA level between the treated and control groups after 7 days. However, the NogoA level tended to be lower in the treatment group than in the control group after 28 days. These results may indicate that ADMSC administration can enhance axonal growth and plasticity by reducing the level of NogoA. In addition to indicating axonal sprouting, these findings are in accordance with the tractography and Cryo-Myelin stain findings that suggest restoration of white tract connectivity in the area of ischemia induced by injection of ET-1.

Conclusions

The findings of this study support the concept that ADMSC play an important role in enhancing some of the major mechanisms of remyelination. ADMSC administration resulted in a smaller lesion size and less cell death, as well as increased cell proliferation including oligodendrocyte progenitors, and higher levels of white matter-associated markers (NF, MBP and Olig-2) and restoration of white tract connectivity in the infarct area. All these processes may help to explain the improvement in functional outcome after ADMSC administration. Therapies that enhance remyelination may help to prevent the functional deficits resulting from those strokes affecting the white matter.

Additional file

Additional file 1: rADMSC proteomic analysis of cell supernatants.

Abbreviations

ADMSC: Adipose tissue-derived mesenchymal stem cells; AU: Arbitrary units; DAPI: 4′,6-diamino-2-phenylindole; DiI: Celltracker CM-DiI; DTI: Diffusion

tensor imaging; ET-1: Endothelin-1; FASP: Filter aided sample preparation; FITC: Fluorescein isothiocyanate; GFAP: Glial fibrillary acidic protein; i.v.: Intravenously; LC-MS/MS: Liquid chromatography tandem mass spectrometry; MBP: Myelin basic protein; MRI: Magnetic resonance imaging; NF: Neurofilament; NogoA: Neurite outgrowth inhibitor; Olig-2: Oligodendrocyte transcription factor 2; PE: Phycoerythrin; ROI: Region of interest; SEM: Standard error of the mean.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
LOO and JRC designed the experiments, performed animal experiments, and participated in drafting the manuscript. BRF was responsible for the laboratory assays. BF participated in coordination and helped in drafting the manuscript. TS and FC participated in cell culture studies and helped in drafting the manuscript. TNH and SC were responsible for the MRI laboratory assays and tractography. JAL and JV performed and analyzed the proteomics experiments. MGF and EDT designed the experiments, participated in coordination and helped in drafting the manuscript. All authors have read and approved the manuscript for publication.

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DISCUSIÓN

En los artículos anteriores ha quedado expuesto el potencial terapéutico de la administración de factores tróficos así como la terapia celular con el fin de estimular la reparación tras el infarto cerebral. Estos trabajos demuestran cómo la administración intravenosa del factor neurotrófico del cerebro (BDNF), así como de células troncales mesenquimales de tejido adiposo (CTM-TA), mejoran la recuperación funcional en el modelo animal de infarto cerebral subcortical. Ambas estrategias son capaces de potenciar la recuperación funcional a través de mecanismos que incluyen la estimulación de la proliferación de células precursoras de oligodendrocitos, la reparación de las fibras de axón y mielina, así como promover un aumento de la conectividad cerebral. Se sugiere, a su vez, cómo el mecanismo de acción de las CTM-TA podría estar mediado por la liberación de factores tróficos y de señalización al medio extracelular. Mediante los estudios de proteómica, se han podido identificar más de 2.400 factores producidos por las CTM-TA, incluído el propio BDNF, cuya actividad en conjunto podría ser la responsable de la buena recuperación funcional observada. Estudios futuros deberían comparar ambas estrategias (BDNF y CTM-TA), con el fin de identificar cuál de las dos podría ser de mayor interés de cara a su traslación a pacientes de infarto cerebral con afectación subcortical.

A la vista de los resultados expuestos, queda clara la necesidad de desarrollar nuevas estrategias terapéuticas dirigidas a la protección y reparación del cerebro en su conjunto, incorporando el estudio de la afectación subcortical, así como estrategias para la reparación de las fibras nerviosas de axón y mielina. Gracias a la investigación en infarto cerebral conocemos en mayor profundidad los mecanismos patogénicos que acontecen a nivel molecular durante la isquemia y reperfusión. Sabemos hoy, que en el infarto cerebral la intervención durante la fase aguda es crucial para evitar la progresión de los mediadores de daño de la cascada isquémica y salvar la mayor cantidad de tejido cerebral posible. A pesar de los esfuerzos en investigación traslacional, el único tratamiento disponible continúa siendo la reperfusión con rtPA, que sólo está disponible en un número limitado de pacientes debido a sus contraindicaciones y estrecha ventana terapéutica. Por

ello, la búsqueda de nuevas estrategias terapéuticas que favorezcan no sólo la protección, sino también la reparación del tejido dañado, a través de la estimulación de procesos como la neurogénesis, oligodendrogénesis, sinaptogénesis, angiogénesis, así como la regeneración axonal y la remielinización, es fundamental. Teniendo en cuenta la alta incidencia que tiene la afectación subcortical en los pacientes con infarto cerebral, es de alto interés desarrollar estrategias terapéuticas que permitan potenciar la reparación a nivel de las fibras nerviosas. Los resultados de los trabajos aquí expuestos sugieren que tanto el BDNF como las CTM-TA podrían ser estrategias eficaces para la reparación de las mismas en los pacientes de infarto cerebral con afectación subcortical.

Hasta ahora, la mayoría de modelos animales de infarto cerebral reproducen únicamente el daño cortical o cortico-subcortical, por lo que en los últimos años diferentes laboratorios han hecho esfuerzos para desarrollar nuevos modelos que permitan estudiar el daño sobre las fibras de sustancia blanca (axón y mielina). Este es el caso de los modelos centrados en la lesión de fibras del cuerpo calloso [52]. Sin embargo, en estos modelos el daño producido es leve y la falta de déficit funcional en los animales limita su interés desde un punto de vista traslacional, puesto que no se corresponde con el daño observado en la mayoría de pacientes que muestran afectación subcortical. En este trabajo de Tesis Doctoral, se puso a punto el modelo de infarto subcortical por inyección de Endotelina-1, en el que sí observamos déficit funcional motor cuantificable, y que por tanto constituye un modelo de interés traslacional para la búsqueda de estrategias como el BDNF y las CTM-TA, enfocadas a la reparación de fibras nerviosas.

1. RECUPERACIÓN FUNCIONAL Y BIODISTRIBUCIÓN TRAS LA ADMINISTRACIÓN DE BDNF Y CTM-TA EN EL INFARTO CEREBRAL SUBCORTICAL.

Bajo una perspectiva traslacional, es importante analizar en los animales el efecto de los tratamientos sobre las disfunciones motoras que típicamente se observan en los pacientes de infarto cerebral con afectación subcortical. En trabajos anteriores, ya había sido descrito cómo el test de la barra longitudinal [54], el del cilindro rotatorio [55], así como la escala modificada de Rogers [101], permitían la valoración de los déficits motores, alteraciones típicas que observamos en estos pacientes. A la vista de los resultados expuestos, tanto la administración de BDNF como la de CTM-TA son capaces de promover la recuperación funcional en los animales tras infarto cerebral subcortical. Estudios previos habían demostrado cómo la administración de BDNF intravenoso era capaz de promover la recuperación funcional en el modelo animal de infarto cerebral cortical inducido fotoquímicamente [64]. Del mismo modo, la administración de CTM-TA mostró buenos resultados sobre la recuperación funcional en modelos animales en estudios de nuestro laboratorio y de otros grupos [55, 82]. Sin embargo, hasta ahora no había información respecto a la capacidad de ambas estrategias terapéuticas para potenciar la recuperación funcional tras el daño subcortical. Los resultados de nuestro estudio muestran cómo ambas estrategias terapéuticas (BDNF y CTM-TA) promueven la recuperación funcional tras infarto subcortical en los animales para cada uno de los tests funcionales empleados, sugiriendo que podrían ser estrategias capaces de promover la reparación del daño sobre las fibras nerviosas en aquellos pacientes de infarto cerebral que presentan afectación subcortical. Futuros estudios deberán analizar el efecto de distintas dosis de tratamiento en los animales, con el fin de identificar si hubiera una dosis de tratamiento mínima efectiva para cada una de las aproximaciones terapéuticas. Por otro lado, deberán realizarse estudios comparativos con el fin de estudiar cuál de las aproximaciones terapéuticas (BDNF ó CTM-TA) es más ventajosa sobre la recuperación funcional. Como limitaciones a considerar para futuros trabajos, los tests funcionales

empleados en los estudios en animales típicamente evalúan la capacidad motora, mientras que es sabido que en los pacientes de infarto cerebral también se observan déficits de memoria y alteraciones de tipo cognitivo [102, 103]. En este sentido, los estudios futuros en animales deberán analizar el efecto de los tratamientos sobre este tipo de alteraciones menos estudiadas.

Respecto a las vías de administración, en este estudio de seleccionó la intravenosa ya que el uso de rutas poco invasivas es fundamental de cara a la traslación a pacientes. Estudios previos habían sugerido que el BDNF era capaz de atravesar la barrera hemato-encefálica (BHE) cuando éste era administrado de forma sistémica [64, 104]. Al analizar en nuestro estudio la biodistribución del BDNF, los resultados mostraron un aumento significativo del factor trófico en el cerebro de los animales ya a las 4 horas tras su administración, confirmando que éste era capaz de atravesar la BHE en nuestro modelo y llegar al cerebro isquémico. En cuanto al mecanismo de acción, el aumento de BDNF en el cerebro de los animales parecía el responsable del efecto terapéutico observado, ya que otros trabajos habían mostrado cómo el BDNF participa activamente en el control de los circuitos neuronales, la plasticidad dendrítica y el crecimiento axonal en las áreas limítrofes al infarto [64, 105-107]. Respecto al estudio del efecto del tratamiento con CTM-TA, no observamos, sin embargo, la llegada de células al cerebro de los animales al ser administradas por vía intravenosa. Estos resultados van en la misma línea de trabajos previos de terapia celular de nuestro grupo de investigación, en los que ya había sido descrito cómo no era necesaria la llegada de las células al cerebro lesionado para obtener una buena recuperación funcional tras su administración sistémica [55, 56]. A pesar de que todavía algunos estudios siguen centrados en la inyección intracerebral de las células [108], nosotros optamos por la administración intravenosa, ya que es menos invasiva y más aceptable desde un punto de vista traslacional. Aunque algunos estudios habían descrito cómo las células pueden atravesar la BHE tras su administración sistémica por encontrarse ésta comprometida tras el infarto cerebral en fase aguda [77, 81, 109], en

nuestro estudio no observamos implantación de las células en el cerebro de los animales, quedando éstas retenidas en órganos periféricos como el bazo, pulmón e hígado, en consonancia con resultados previos de nuestro grupo de investigación y de otros [55, 56, 110, 111]. Numerosos mecanismos se han propuesto para explicar el mecanismo de acción de las CTM-TA, como son la transdiferenciación celular, la inmunomodulación y la liberación de factores tróficos y otras moléculas señalizadoras [75, 76, 97]. Como transdiferenciación celular, se entienden todos aquellos procesos que implicarían la diferenciación de las células administradas a neuroblastos o precursores de células neurales en el cerebro lesionado. Algunos estudios han mostrado que esto es posible [112, 113], sin embargo debido al escaso número en que éstas son capaces de llegar al cerebro, así como las condiciones de estrés imperantes en el área dañada, el anidamiento a largo plazo, y sobre todo la integración de las pocas células diferenciadas en circuitos neuronales funcionales se torna prácticamente impensable. De hecho, estudios en paralelo realizados en el laboratorio en los que las células son administradas por vía intracarotídea, muestran cómo a los 14 días, las células colocalizan con marcadores de células fagocíticas como CD11b, indicando que podrían haber sido incorporadas por dichas células para ser eliminadas del sistema nervioso [86]. El segundo mecanismo atribuído a las CTM es la inmunomodulación, la cual podría deberse tanto a la liberación de factores inmunomoduladores tipo citoquinas por parte de las CTM [114], como al efecto inmunomodulador que podría tener el propio reconocimiento de las CTM por parte del sistema inmune del huésped. A pesar de no producir un “rechazo” clásico al carecer del complejo mayor de histocompatibilidad de tipo 2 (CMH-II), la interacción en dicho reconocimiento antigénico podría tener efectos inmunomoduladores con implicaciones sobre la progresión y recuperación del infarto. Finalmente, el mecanismo que más interés ha suscitado en los últimos años es la liberación por parte de las CTM de factores tróficos y sustancias señalizadoras, que podrían modular tanto la progresión de los distintos eventos de la cascada isquémica, como los mecanismos de reparación cerebral: neurogénesis,

oligodendrogénesis, sinaptogénesis, remodelación axonal y angiogénesis [55]. Pero, ¿a través de qué mediadores concretos son capaces de actuar las CTM-TA cuando son administradas por vía intravenosa y retenidas en los órganos periféricos? Ya había sido descrito con anterioridad cómo las CTM-TA producen citoquinas, factores de crecimiento y otros factores solubles capaces de promover la proliferación, la diferenciación celular así como los cambios necesarios para la correcta remodelación tisular [97], [76, 94, 115], e incluso algunos grupos han sugerido previamente cómo el efecto terapéutico de las CTM-TA podría estar mediado por la liberación de factores tróficos [116]. En este trabajo tuvimos la oportunidad de estudiar por proteómica el sobrenadante de las células en cultivo con el fin de identificar qué factores estaban produciendo al medio extracelular. Los resultados de este estudio permitieron identificar más de 2.400 factores secretados *in vitro* por las CTM-TA, entre ellos el propio BDNF, los cuales podrían estar ejerciendo una función paracrina una vez que las células quedan retenidas en los órganos periféricos tras su administración por vía sistémica. Sin duda, la relación entre estos efectos paracrinos y la buena recuperación funcional observada deberá ser estudiado en profundidad en futuros estudios, así como el estudio del papel de cada uno de los factores identificados, con el fin de entender en detalle el mecanismo de acción de las CTM-TA en el infarto cerebral.

2. EFECTOS DE LA ADMINISTRACIÓN DE BDNF Y CTM-TA TRAS INFARTO CEREBRAL SUBCORTICAL. MECANISMOS DE ACCIÓN

2.1. Tamaño de lesión y conectividad cerebral

Estudios previos en modelos animales ya habían mostrado cómo la administración de factores tróficos como el VEGF o EPO estaban asociados a una reducción del volumen de lesión tras infarto cerebral [60, 117]. Sin embargo, la mayoría de estos estudios estaban centrados en el daño cortical, y hasta ahora la información acerca del efecto terapéutico que los factores tróficos podrían tener sobre la recuperación tras el daño en fibras

nerviosas era muy limitado. Respecto al BDNF como factor trófico, ya había sido probado en modelos animales de infarto cerebral, y los efectos del mismo sobre la reducción del tamaño de lesión había dado resultados dispares. En un primer estudio del grupo de Schäbitz y colaboradores, en el que se empleó el modelo de oclusión transitoria de la arteria cerebral media mediante ligadura, se observó una reducción de este parámetro a las 24h tras el infarto cerebral a una dosis de 900ug/kg [63]. Sin embargo, estudios del mismo grupo, en el que los autores analizaron el efecto de la administración del BDNF en dosis consecutivas de 20ug durante 5 días tras infarto cerebral inducido fotoquímicamente (100ug en total), los autores no encontraron una reducción significativa de dicho parámetro [64]. Aunque sería interesante ver si las diferencias entre ambos estudios pudieran ser debidas a la dosis, los resultados de ambos estudios no son comparables, ya que los modelos animales de infarto cortical utilizados no fueron los mismos. Nuestro estudio era, por tanto, el primero en analizar el efecto de la administración del factor trófico en la lesión subcortical. Nuestros resultados muestran cómo la administración de BDNF no estuvo asociada a una reducción en el tamaño de lesión ni a los 7 ni a los 28 días. Sin embargo, tal y como muestran los resultados de conectividad cerebral, a pesar de no observarse una reducción en el tamaño de lesión en las imágenes en T2, sí se observó una mejoría en la conectividad cerebral en el grupo de tratamiento, tal y como mostraron los resultados de la tractografía a los 28 días, resultados que pudieron ser confirmados tras el sacrificio de los animales mediante inmunohistoquímica específica para mielina por la técnica de Cryomyelin. El hecho de no haberse observado diferencias en el tamaño de lesión en las imágenes en T2, y sí en los mapas de conectividad, enfatiza la importancia de incorporar este tipo de técnicas a los estudios en modelos animales.

Respecto al estudio del efecto de la administración de las CTM-TA, trabajos previos en modelos animales habían mostrado una reducción en el tamaño de lesión tras el tratamiento con CTM. No obstante, la mayoría de estos trabajos han estado centrados en

lesión cortical [82, 83, 118]. Además, se trata de estudios en los que las células administradas han sido modificadas genéticamente para la expresión constitutiva de factores tróficos [119-122]. Los resultados mostraron una reducción en el tamaño de lesión a los 28 días tras la administración de las CTM-TA tras infarto cerebral subcortical. Estos resultados contrastan con estudios previos llevados a cabo en nuestro grupo de investigación, en los que la mejoría funcional y el tamaño de lesión eran evaluados a los 14 días tras el tratamiento y no se encontraba una reducción significativa del tamaño de lesión, a pesar de la buena recuperación funcional [55, 56]. Así, los hallazgos a los 28 días sugieren la importancia de analizar el efecto de las CTM-TA a tiempos más largos. De hecho, al analizar los resultados de los mapas tractográficos *in vivo*, se observó que el tratamiento con CTM-TA estaba asociado a una mejoría de la conectividad cerebral en los animales a los 28 días, que pudo ser confirmada tras el sacrificio mediante la técnica Cryomyelin.

Los resultados de este trabajo, muestran la importancia de realizar estudios de neuroimagen a distintos tiempos tras los tratamientos. Puesto que en ambos estudios (BDNF y CTM-TA) observamos una mejoría de la conectividad cerebral a los 28 días del tratamiento, pero en el caso concreto de las CTM-TA observamos además, una reducción en el tamaño de lesión, futuros estudios comparativos son de interés, con el fin de confirmar si efectivamente el tratamiento con CTM-TA pudiera ser más eficaz que el BDNF sobre esta variable de estudio.

2.2. Proliferación celular

Numerosos mecanismos están implicados en la reparación cerebral, entre los que se encuentran la neurogénesis, sinaptogénesis, angiogénesis y oligodendrogénesis [12, 16, 123]. Estudios previos en modelos animales muestran cómo el pico de proliferación celular se encuentra dentro de los 5 y 7 primeros días tras la isquemia cerebral [124]. Como nichos neurogénicos en el cerebro adulto, que pueden ser además estimulados tras

el daño, se conocen la zona subgranular del hipocampo, la ZSV y el bulbo olfatorio [125-129]. Sabemos también que después del infarto, las células precursoras proliferantes migran hacia la zona de peri-infarto para la formación de nuevos vasos con el fin de restablecer el aporte de oxígeno al área cerebral afectada [39]. La formación, sin embargo, de nuevas células precursoras de oligodendrocitos tras la lesión, así como la maduración de las mismas y la formación de nuevas fibras nerviosas o la inducción de la remielinización tras infarto, han sido procesos menos estudiados [23]. El estudio de los oligodendrocitos es de hecho de alto interés, ya que son las células responsables de la formación de mielina a lo largo de los axones maduros, y ha sido descrito cómo son células especialmente vulnerables al daño isquémico [34, 130].

Los resultados del grupo de Schäbitz y colaboradores ya habían descrito cómo la administración del BDNF era capaz de potenciar la proliferación y formación de nuevos neuroblastos en el modelo animal de infarto cerebral cortical [64]. Nuestros resultados van en la misma línea, ya que observamos cómo la administración del BDNF está asociada a un aumento significativo de la proliferación celular a los 7 días en el hemisferio ipsilateral a la lesión. Quisimos a su vez analizar si dicha proliferación celular estaba asociada a la proliferación de oligodendrocitos y a la reparación de las fibras de sustancia blanca. Las células encargadas de la formación y maduración de las mismas son las CPO, originadas en la ZSV y presentes en prácticamente todo el parénquima cerebral. Estas células pueden ser identificadas mediante microscopía ya que son positivas para los marcadores A2B5, CNPase, APC y PDGFR. Nuestros resultados mostraron cómo las células proliferantes (KI-67 positivas) que observábamos a los 7 días en la ZSV, colocalizaban con cada uno de los marcadores de CPO mencionados anteriormente (A2B5, CNPase, APC y PDGFR). De este modo, confirmábamos cómo la administración de BDNF potenciaba la oligodendrogénesis en los animales tratados, sugiriendo que el efecto terapéutico que observamos en los animales tratados con BDNF podría ser debido a dicha estimulación glial. Respecto al estudio del efecto de la administración de CTM-TA, ya había sido descrito

en la literatura cómo las CTM inducen un incremento en la proliferación de neuroblastos y otros precursores neurales en la zona de infarto [131]. En nuestro estudio pudimos observar un incremento en el número de células en división en el área de lesión a los 7 días en el grupo de tratamiento, que fue finalmente significativo a los 28 días. Los estudios de colocalización mostraron cómo las células proliferantes KI67+ presentaban marcadores típicos de células astrocitarias (GFAP), neuronales (NeuN), así como de oligodendrocitos (Olig-2), sugiriendo que el efecto de las CTM-TA sobre la recuperación funcional podría ser debido a la estimulación de la proliferación de varias estirpes neurales en los animales tratados de forma simultánea tras el tratamiento.

2.3. Mecanismos de reparación cerebral

Considerando al cerebro en conjunto, la diana terapéutica en el infarto cerebral es la unidad neurovascular, formada por neuronas, astrocitos, microvasos, pericitos, células de soporte y oligodendrocitos, así como la totalidad de fibras nerviosas de axón y mielina. Tal y como ha quedado reflejado, los mecanismos de protección y reparación cerebral pueden ser potenciados mediante la administración de factores tróficos así como mediante la terapia celular [123].

Para la correcta formación de fibras nerviosas, es necesario el desarrollo de células precursoras de oligodendrocitos que una vez diferenciadas formen la vaina de mielina a lo largo de los axones, una vez alcanzada su madurez funcional. Respecto al estudio del efecto de la administración del BDNF, al analizar los niveles de marcadores característicos de CPO en la zona de peri-infarto a los 7 días del tratamiento, tanto por inmunoflorescencia como mediante Western Blot, encontramos un aumento significativo en los niveles de A2B5, CNPase y O4 en los animales tratados con el factor trófico. Ya había sido descrito cómo la vía de señalización del BDNF y su receptor TrkB participan en el daño en sustancia blanca [33], y del mismo modo, en el estudio de Schävitz y colaboradores [64] se mostró cómo el BDNF administrado de forma sistémica era capaz de

estimular la reparación cerebral a través de un aumento de neuroblastos en la zona de peri-infarto. Sin embargo, la estimulación de la oligodendrogénesis no había sido estudiada en este trabajo. Así, habiendo encontrado un aumento significativo de los niveles de marcadores de CPO a los 7 días en nuestro estudio, parecía esperable encontrar un incremento en los niveles de marcadores de oligodendrocitos y fibras nerviosas maduras a los 28 días. Esto fue lo que encontramos al analizar marcadores de fibras como la proteína básica de mielina (MBP en inglés), así como del marcador de oligodendrocitos maduros Olig-2, para los cuales observámos un aumento significativo en los animales tratados con el factor trófico, en comparación con el grupo control. Además, encontramos una reducción en los niveles de la proteína inhibidora del crecimiento axonal Nogo-A. Hasta ahora, la proteína Nogo-A era más conocida en los distintos estudios por el efecto terapéutico que tiene su inactivación farmacológica mediante anticuerpos monoclonales sobre la estimulación de la plasticidad axonal [132-134]. Sin embargo, el papel de la misma como posible marcador de reparación no había sido descrito. En nuestro estudio, la disminución de los niveles de Nogo-A a los 28 días, junto a una buena recuperación funcional y un aumento en los niveles de MBP y Olig-2, parecen reflejar la estimulación de la reparación de fibras nerviosas mediada por el factor trófico. Estos resultados coinciden con los sugeridos en los estudios de neuroimagen y el aumento de la conectividad cerebral observado en los animales tratados con BDNF a los 28 días, tal y como se describió anteriormente.

De manera análoga, en el estudio del efecto del tratamiento con CTM-TA también encontramos un aumento de los niveles de marcadores de fibras nerviosas adultas MBP y Olig-2 en el grupo tratado. En este caso encontramos mayores niveles de MBP tanto a los 7 como a los 28 días. El hecho de que ya a los 7 días los niveles de MBP fuesen mayores en el grupo tratado con CTM-TA, podría ser explicado por la suma de un efecto a la vez protector y reparador de las CTM-TA tras el infarto cerebral que ya ha sido descrito en la literatura [55, 135, 136]. En este trabajo se analizaron además los niveles de la proteína

neurofilamento (NF), componente estructural mayoritario del citoesqueleto de los axones y dendritas. Hoy sabemos que los niveles de este marcador aumentan en la zona de peri-infarto a medida que progresa la reparación cerebral [137-139]. De hecho, ya había sido observado cómo el tratamiento con CTM induce una estimulación de la plasticidad axonal, que se manifiesta, entre otras, en un aumento de los niveles de la proteína NF en modelos animales tras infarto de tipo cortical [55, 140]. En nuestro estudio, los niveles de NF aumentaron a los 28 días tras el tratamiento con CTM-TA, sugiriendo un aumento de la densidad axonal asociado a la buena recuperación funcional que encontrábamos en los animales. De manera análoga al estudio con BDNF, el tratamiento con CTM-TA también mostró una reducción de los niveles de Nogo-A en el cerebro de los animales tratados a los 28 días, aunque dicha reducción no llegó a ser estadísticamente significativa. Los resultados de nuestro estudio demuestran, por tanto, cómo el efecto positivo sobre la recuperación funcional observado tras el tratamiento con CTM-TA podría ser debido a la estimulación de la proliferación celular así como a la preservación y formación de nuevas fibras nerviosas que se manifestaron en un aumento de conectividad cerebral en los animales tratados a los 28 días, resultados que pudieron ser confirmados en los estudios de neuroimagen. Quizás debido a la secreción de múltiples factores tróficos y moléculas señalizadoras de forma simultánea por parte de las CTM-TA, entre ellos el BDNF, los resultados de nuestro estudio sugieren cómo el efecto terapéutico de las CTM-TA actúa de forma simultánea tanto a nivel de protección, como de reparación tras infarto cerebral subcortical. Tal y como se mencionó en la introducción, los procesos de protección y reparación son indivisibles, actuando a modo de continuo a lo largo de la cascada isquémica, y ambos parecen ser potenciados por las CTM-TA cuando son administradas por vía sistémica.

Los resultados de este trabajo demuestran cómo ambos tratamientos (BDNF y CTM-TA) mejoran la recuperación funcional tras infarto cerebral subcortical a través de mecanismos que incluyen la potenciación de la proliferación celular, así como un aumento

de la conectividad cerebral en la zona de lesión. Tras haber demostrado el efecto terapéutico del BDNF y las CTM-TA en el modelo de infarto cerebral subcortical, y habiendo identificado al BDNF como uno de los más de dos mil factores protéicos liberados por las CTM-TA *in vitro*, futuros estudios deberán comparar ambas aproximaciones terapéuticas con el fin de identificar cuál de ellas presenta un mayor potencial para su traslación a los pacientes de infarto cerebral con afectación subcortical.

Los resultados de este trabajo sugieren que ambas estrategias terapéuticas (BDNF y CTM-TA) podrían ser útiles para la reparación del daño subcortical que acompaña a la lesión de pequeño vaso, así como para los infartos cerebrales de gran vaso en que resultan afectados tanto el cortex como el subcortex.

CONCLUSIONES

En un modelo experimental en ratas de infarto cerebral subcortical, observamos:

1. La administración del Factor Neurotrófico derivado del Cerebro (BDNF) mejora la recuperación funcional.

2. La administración del BDNF potencia mecanismos de reparación cerebral (aumento de células progenitoras de oligodendrocitos, incremento en la densidad de fibras de sustancia blanca y conectividad cerebral).

3. La administración de células troncales mesenquimales alogénicas derivadas de tejido adiposo (CTM-TA) mejora la recuperación funcional.

4. La administración de las CTM-TA potencia mecanismos de reparación cerebral (aumento de proliferación celular y de marcadores de sustancia blanca, incremento en la densidad de fibras de sustancia blanca y conectividad cerebral).

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